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(Plant Bioenergetics)
Project Reports (1)

# PROJECT REPORT FORM

PROJECT NO. 2691
COOPERATOR Institute of Paper Chemistry
REPORT NO. 1
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SIGNED M. A. Johnson

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Summary

Five preliminary experiments utilizing whole cabbage mitochondria (and whole chloroplasts in one instance) were conducted. The first three experiments yielded very little useful information due to the fact that the phosphorylation activity by the suspensions of subcellular particles was very low and exhibited poor replication. The lack of activity by the particles was at least partially traceable to heating during the homogenization process. Since only a colorimetric assay for the disappearnce of inorganic phosphate (Pi) was employed, the data obtained in these cases of low Pi turnover was not reliable. Therefore, a procedure whereby chloroplasts and mitochondria can be isolated during the same operation has not yet been developed. Sufficient Pi turnover did occur in experiments No. 4 and No. 5 to allow some conclusions. The experimental conditions used in all five preliminary experiments were probably suboptimal either due to lack of some essential cofactor and/or because the glycylglycine buffer used was partially inhibitory. Results indicate the latter possibility to be the major factor. The addition of cofactors such as nicotinamide adenine dinucleotide ($NAD^+$) and coenzyme A (CoA) elicited some improvement in Pi turnover (experiment No. 4). Results obtained with a new buffer, morpholinopropane sulfonic acid (MOPS) were very encouraging (experiment No. 5) and indicate that glycylglycine should be replaced by MOPS in much of this work. Note that dithiothreitol behaved differently in the two buffer systems.

The single attempt (experiment No. 6) to isolate the active intermediate(s) of oxidative phosphorylation from cabbage mitochondrial acetone powder was successful. $^{32}P$ was used in the endeavor as usual. The use of glycylglycine buffer versus MOPS and the presence or absence of $NAD^+$ during assay were the variables. The previously observed stimulatory effect of $NAD^+$ upon the transfer reaction was confirmed. MOPS appeared to be a better buffer for the transfer reaction in the absence of $NAD^+$ but an inhibitor of the $NAD^+$ stimulation of transfer. The observed effect of MOPS in the absence of $NAD^+$ may contain a very minor element of quantitative uncertainty since it presupposes the ability to make two identical mitochondrial preparations. There is little doubt that this would constitute a very small correction, if any, which could be obtained by a protein determination. However, visually, the MOPS preparation appeared to be equivalent or, if anything, smaller than the glycylglycine preparation. Since the improvement of MOPS over glycylglycine was substantial (>2 1/2 fold), it was a real effect which may well be one of increased intermediate stability in the presence of MOPS. One can safely conclude that MOPS is at least equivalent to and probably much better than glycylglycine in this phase of the research also. Since MOPS is less expensive and has a pKa (7.2) closer to neutrality than glycylglycine (8.1), it will be used in a routine manner replacing glycylglycine in the future. Because of the behavior of $NAD^+$ stimulation relative to MOPS, glycylglycine may still be useful and necessary in attacking this aspect of the problem.

Progress on the project has taken the form of reestablishing links with research which has lain idle for two years. At the present time the continuity of the research effort has been largely regained and a new buffer system shows good promise of providing a superior milieu for assay as well as exerting

what may prove to be a rather specific effect upon $NAD^+$ stimulation of transfer.

Results (All results are averages of triplicates.)

Experiment No. 4

Whole Mitochondria

| ADP-Hexokinase-Glucose | $NAD^+$ + CoA | ΔPi (μmoles/50 min.) |
|---|---|---|
| + | - | -8.7 |
| + | + | -11.0 |

Experiment No. 5

Whole Mitochondria

(ADP-Hexokinase-Glucose-$NAD^+$-CoA in all flasks)

| Buffer | Dithiothreitol | ΔPi (μmoles/90 min.) |
|---|---|---|
| Glycylglycine | - | -2.3 |
| | + | -6.4 |
| MOPS | - | -44.2 |
| | + | -31.9 |

Experiment No. 6

Acetone Powder Extract

($^{32}P$ Assay at $10^{-4}$M Pi)

| Buffer | $NAD^+$ | Pi Incorporation (mμmoles/20 min.) |
|---|---|---|
| Glycylglycine | - | 0.20 |
| | + | 0.73 |
| MOPS | - | 0.53 |
| | + | 0.55 |

Outlook

The preliminary experiments with whole mitochondria and chloroplasts

indicate that when glycylglycine is the buffer $^{32}P$ is needed to eliminate uncertainties in the results. MOPS has not been tried for chloroplasts, but, if mitochondrial reaction is any indication, glycylglycine may be replaced by MOPS in chloroplast isolation also. Pi turnover in the presence of MOPS is sufficient so that $^{32}P$ need not be used if undesirable for any reason. Activity and replication might be improved by the addition of another cofactor, thiamin pyrophosphate (TPP). TPP has been employed routinely in much previous whole cabbage mitochondria research, but it was not available during the recent experiments (on order since December, 1967). Investigations on whole mitochondria and chloroplasts are not crucial in order to proceed with the intermediate studies and, as such, are of rather low priority at the moment. Another attempt may be made in the near future to prepare mitochondria and chloroplasts at the same time using $^{32}P$ and MOPS instead of glycylglycine. It is hoped that we may have some electron micrographs of these preparations before long also.

The major branch of the investigation, on the oxidative phosphorylation intermediate, will aim toward purification soon. However, such endeavor must be preceded by a test of stability in the new MOPS system. As stated earlier, there is a hint that the intermediate has increased stability in MOPS. Limited data obtained previously on this problem in glycylglycine shows that the intermediate has a half-life of 7-8 hours at 0-4°. This dramatizes the fact that stabilization is a first* order of business, for, unless one has an extremely rapid purification procedure, the activity will be undetectable (gone?) by the time one is ready to determine the outcome of a purification step.

*It may be worthwhile to attempt a separation such as disk electrophoresis soon to get an idea of the number and mobility of proteins one is dealing with in the crude extracts.

This may be a major stumbling block in these investigations, i.e., how to purify something which doesn't stick around long enough to be manipulated. Buffer change may be a means of stabilization, but other approaches using solvents and chemical additives may be tried. It would be nice to know if the instability so far observed is due to simple chemical hydrolysis or if the hydrolysis is enzymatic. It is conceivable also that the inactivation is not the manifestation of a hydrolytic reaction at all. If gathering information on the causes of inactivation were not such a formidable task in itself, one would first seek this knowledge which would in turn dictate which stabilization approaches would be most apt to be fruitful. For example, normally a simple heat treatment could distinguish whether an enzymic reaction was involved in inactivation but, for obvious reasons, that would fail here. Nevertheless, should a number of "intelligent guesses" fail to bring about stabilization, it may be necessary to attack this problem by probing the causes of inactivation. If the inactivation is promoted by other proteins in the extract, then purification itself may lead to increased stability.

# PROJECT REPORT FORM

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## Introduction

A total of eleven experiments employing mitochondrial extracts have been performed since the last report. The duration of these experiments varied from weeks to months depending upon their complexity. Investigations have covered stability, solubility, heterogeneity, fractionation, product identification, reaction rates, fate of $^{32}P$ label, and electron microscopy of preparations. A few significant improvements of routine procedures have been achieved also. While there have been periods of frustration, progress has been made, sometimes in the form of learning what not to do. Results will be presented below in essentially historical order, for, even though this may not be the neatest package, it provides perspective for the project. All tables and figures are located together in the back of the report; in many cases more detail is available than is presented.

## Results and Discussion

An attempt was made to grow friable cabbage tissue in tissue culture; the attempt failed although differentiated tissue grew quite well in one case. Had the growth of friable tissue succeeded, the near absence of fiber and chlorophyll might have made these cultures an attractive source of mitochondria for this project since harsh treatment of the tissue should not be necessary to extract cellular organelles. Although tissue culture is not ruled out, a special effort, which would appear to be necessary for success, is not warranted since whole cabbage remains satisfactory at this stage.

Electron micrographs of "heavy" and "light" cabbage mitochondria used in this research were prepared by Olga Smith; little difference was noted between these two mitochondrial fractions (Plate I). Both fractions are contaminated to some extent with chloroplast fragments (grana) and other organelles which are thought to be lysosomes (or, possibly, degraded mitochondria) and amyloplasts. Thus, although the vast majority of the isolated material is undoubtedly mitochondria (Plate II), at a more refined stage in the research it may be desirable to employ "cleaner," more tediously prepared mitochondria than are justified now. The possibility always exists that the observed intermediate transfer activity is originating from contaminants and not from mitochondria, but it should be pointed out that a preparation that appears to contain only mitochondria in the electron micrographs is still not completely free from this possibility. Therefore, the rigorous exclusion of contaminants as a possible source of activity is given low priority at the present time. The actual number of wash steps, which should produce "cleaner" preparations in direct proportion to the number of washes (based upon the research of others in this field), has not been observed to have any great influence on the biological activity under study. If contaminants were responsible for the activity, then more thoroughly washed preparations should be less active unless the contaminant can't be removed by washing. No systematic study of this hypothesis has been undertaken.

In project report no. 1, instability of the acetone powder extract preparations was described. It was hoped at that time that the use of the new MOPS (morpholinopropane sulfonate) buffer might aid in stabilization. Experimental results failed to bear out this expectation since most of the activity

did not survive one day at 4° in this system (Table I). Further attempts at stabilization consisted of purification as discussed later.

It was desirable to run physical tests on the acetone powder extracts in order to obtain some estimate of the number and size of the extract components. This information allows one to make some assessment of the magnitude and difficulty of the purification he is facing. The sedimentation pattern of freshly prepared mitochondrial acetone powder extracts in the analytical ultracentrifuge showed a small leading peak followed by a more slowly sedimenting, larger, broad peak. These two peaks sedimented at approximately 7.6 S and 4.3 S respectively. Aging the extract a day at 4° or freeze-drying and reconstituting the extract tended to result in patterns with the same two peaks although the 4.3 S peak decreased in size. From these sedimentation data it appeared that the extract contained a number of components, all of which could be classified in one of two sedimentation groups. Therefore, one might expect that separation of the two main groups by size discrimination would be relatively easy compared to the task of separating those components which sedimented together.

Components of similar size might be separated due to differences in electrophoretic mobility under defined conditions. Two attempts were made to disk electrophorese crude extract using a preparative apparatus from which effluent could be continuously monitored with the Uvicord and collected as fractions for assay of intermediate transfer activity. The first trial run was conducted with supernatant decanted from an aged, partially denatured extract. (It is unlikely that the intermediate transfer activity was present in this supernatant but contaminants, from which it must be separated, were present). The electrophoresis resolved two ultraviolet absorbing peaks. The first to

appear had a shoulder on the leading edge; the second peak was well separated and corresponded to the emergence of a visible yellow band. No attempt was made to assay fractions for intermediate transfer activity in this run; however, an experiment followed in which a fresh extract was prepared expressly for preparative disk electrophoresis. The Uvicord trace with fresh extract was somewhat different from that with the aged extract used in the trial run. The leading peak was small (fraction I) while the second peak (fraction III) had a leading slope (fraction II). The yellow band observed in the previous run emerged much later with no detectable ultraviolet peak associated with it. Staining of the gel revealed three bands which had not left the gel and had probably denatured in the gel column. No intermediate transfer activity was evident in fractions I, II, or III in this run, but the control activity of the original unfractionated extract was very weak when assayed at the same age as the three fractions. It was concluded that judgment on the utility of preparative disk electrophoresis should be reserved until it can be evaluated again under conditions in which the unfractionated extract exhibits greater stability toward aging.

A large experiment was begun in early April from which the complete results were not available until mid-May. This experiment had multiple objectives which included the following: (1) Demonstration with unequivocal data of the termination of the intermediate transfer reaction in a short time using high speed (105,000 x g for 14 hours) supernatant. The use of supernatant, obtained after centrifuging the crude mitochondrial acetone powder extract as indicated, would show that the intermediate transfer activity observed had been solubilized and was not due merely to some organized submitochondrial particles still capable

of oxidative phosphorylation. A real intermediate should phosphorylate ADP independently of any simultaneous coupling to an electron transfer process. An intermediate by definition is likely to be a transient species with a high turnover rate. Thus, the high rate of oxidative phosphorylation and other observations in whole mitochondria demand that an intermediate of the process isolated therefrom should also form ATP rapidly; exactly what rates to expect of the isolated system has been the subject of considerable debate in the literature. Since some of the rate-limiting factors are known in the case of the cabbage intermediate transfer activity, it may be possible to approach a rate compatible with the observations in whole mitochondria. Some success occurred earlier with crude extract which has been shown to terminate in five minutes under certain conditions; however, there are complications with crude extract in addition to the main reason for employing high speed supernatant cited above. (2) Examination of the high speed pellet by electron microscopy. If there are submitochondrial particles in crude extract, they should sediment during the high speed centrifugation. Therefore, the appearance of the sedimented material was of interest. Past experiments with shorter centrifugation times have resulted in the formation of pellets which did not possess intermediate transfer activity. (3) Paper chromatography of the products of the intermediate transfer reaction with the hope of demonstrating that glucose-6-$^{32}$phosphate is formed. The assay system used for the intermediate transfer reaction concludes with the measurement of $^{32}$P-labeled organic phosphate. The process of oxidative phosphorylation in whole mitochondria results in the formation of ATP which is converted to glucose-6-phosphate (G-6-P) upon the addition of the enzyme, hexokinase. The reaction products are processed in a manner which results in the isolation and measurement of organic phosphate; this organic phosphate has been shown to be

G-6-P in this system, so it is routinely measured with this assumption. The same assay is used for the intermediate transfer reaction based on the same assumption. However, because of the low phosphate turnover in this reaction, it must be demonstrated that at least some of the labeled organic phosphate measured is G-6-$^{32}$P and not merely some other compound normally unnoticed because of its very low concentration. Since paper chromatography is one of the most common means of resolving phosphorylated sugars, it was the initial choice for the isolation of G-6-$^{32}$P. (4) Investigation of possible $^{32}$P labeling of the high speed supernatant protein followed by analytical disk electrophoresis to localize label and determine the elctrophoretic heterogeneity of the supernatant. The analytical ultracentrifugation mentioned earlier had resolved with certainty only two components in crude extract although more were suspected. The preparative disk electrophoresis of crude extract located two ultraviolet absorbing components plus three additional components which did not emerge from the gel. Thus, the analytical disk electrophoresis had the potential to locate more components which might have escaped detection previously. If any of the components could acquire label due to incubation of the supernatant with $^{32}$Pi in the absence of acceptor (ADP), there was the possibility that one would have isolated a phosphorylated form of the intermediate which should, in theory, exist. On the other hand, a $^{32}$P-labeled component might have been formed from AT$^{32}$P after the transfer reaction was completed or by some other pathway; in that case it would be irrelevant except for the fact that it would indicate the possible amount of competition for AT$^{32}$P experienced by hexokinase in the assay system.

Objective number one was not realized despite trends in the right direction. The fact that these data were somewhat erratic and, therefore, incon-

clusive qualifies the results pertaining to the other three objectives. The high speed pellet was examined by electron microscopy (Plate III) and was found to contain a few membrane fragments plus some intact double membrane units which have no readily identifiable internal structure. Although one cannot judge solely by the appearance of electron micrographs, they are structures which should be considered potential sources of intermediate transfer activity despite the fact that similar pellets have been shown to be inactive in the past. The desirability of removing them from the extract is probably satisfied routinely now by the gel filtration of crude extract which is discussed later. The paper chromatography of reaction products was also inconclusive. A major chromatographic problem was that, without conducting the usual molybdate extraction to remove orthophosphate, much of the label was found in unidentified compounds which did not move on the paper chromatogram. There was some $^{32}P$ count in the G-6-P region of the chromatogram, but one could at best say that G-6-$^{32}P$ was still a possibility as a reaction product because the radioactivity did not peak and the separation of orthophosphate and G-6-P was marginal.

In the phase of the experiment dealing with objective number four, 10 ml portions of high speed supernatant were incubated at 4° for 30 minutes with Pi-$^{32}$Pi in various combinations with $NAD^+$ and $MgCl_2$ (Table II). After incubation each incubation mixture was passed through a Biogel P6 column equilibrated with buffer. The effluent from the columns was monitored with the Uvicord and, in each case, an excluded peak (presumably protein) was obtained followed by a large included peak (Figure II). The excluded peaks from Biogel P6 were analytically disk electrophoresed in each case. One can see from the staining results in Figure I that treatment A gave rise to the most bands and also had the greatest count of the whole gels (Table II). Treatment B gave two

fewer bands of high mobility than did A while treatments C and D gave results similar to each other, patterns with three fewer bands of high mobility than treatment A. It is suggested that the presence of $NAD^+$ and $MgCl_2$ may either prevent dissociation or promote aggregation of proteins in the mixture. Fractions cut out and counted showed peaks of radioactivity in every case as can also be seen in Figure I. If it could be shown in the future that the label in one of these peaks will transfer to ADP, one would have evidence for the isolation of a phosphorylated intermediate as mentioned earlier.

The large ultraviolet absorbing peak included on Biogel P6 from treatment E was subsequently passed through a Biogel P2 column to fractionate it further. This included fraction from Biogel P6 was considerably more radioactive than the excluded protein peak (Figure II), and it was hoped that some clue might be obtained as to what was acquiring label from $^{32}Pi$, or at least its approximate size. The effluent from Biogel P2 was separated into almost three hundred fractions of which those of interest were then recombined into four fractions and freeze-dried. The recombinations were F-1 (1-18), F-2 (19-40), F-3 (41-57), and F-4 (58-170). On the Uvicord trace there were minor peaks at fractions 1, 34, and 86 and a major peak at fraction 48. The majority of counts were in fractions 1-18 followed by trailing of the count except for the small shoulder around fraction 30 (Figure III). Figure III also shows that a very small amount of material accounted for the ultraviolet absorption of the Biogel P6 included peak. The recombined fractions were analyzed for infrared absorption, ultraviolet absorption, and, for F-4 only, semiquantitative emission spectra. F-1 and F-2 both had infrared spectra essentially identical to the MOPS buffer. Why the $^{32}P$ travels with it is not understood, but it should be noted (Figure III) that there is some other material in these

fractions as indicated by the ultraviolet absorption. It was suggested by the analytical department that F-3 and F-4 were largely inorganic in nature; therefore, the ultraviolet absorption of F-3 must be due to a small fraction of the 1 mg of material in the peak area.

An experiment followed this work in which the stability of the intermediate transfer activity during the period of overnight high speed centrifugation was ascertained. It was found that the activities of crude extract and high speed supernatant, when both were sixteen hours old, were nearly identical. However, in both cases only about 25% of the original activity remained. Inspection of the results in Figure IV reveals the problem of percentage comparisons based on a changing control. Recent modification of the isobutanol-benzene extraction procedure, discussed later, should reduce this problem.

The foregoing results were consistent with the conclusion that the intermediate transfer activity is soluble, i.e., it does not sediment upon prolonged centrifugation at 105,000 x g. Nevertheless, the stability problem remained. Also troublesome was the erratic data obtained from the isobutanol-benzene extraction procedure. In an effort to avoid the extraction problem, a different assay for the hypothesized product (ATP) of the intermediate transfer reaction was attempted based on the ATP-specific firefly luciferase. This assay system has not worked, principally because of myokinase which accompanies luciferase in the firefly lantern extracts. The ATP produced by myokinase by dismutation of ADP swamps out any ATP produced by the intermediate transfer reaction. A luciferase system would have to be completely purified of myokinase to be used reliably.

Since column work with Biogels P6 and P2 had revealed some limited information as to the composition of the acetone powder extract, it was decided to forge

ahead with a purification procedure which might simultaneously confer stability upon the intermediate. Since high speed centrifugation for several hours could not be tolerated, an attempt was made to initiate purification as soon as possible after the crude extract was prepared. The low molecular weight materials found by fractionation with Biogels P6 and P2 were possible causes of instability. Thus, in an effort to separate quickly the low molecular weight components from the protein, the acetone powder was extracted with Biogel P6 equilibrated with a slight excess of buffer. The extract containing Biogel P6 beads was then placed immediately on a Biogel column which would exclude and rapidly elute the protein (presumably the active fraction) and leave the unwanted low molecular weight materials behind. At first a Biogel P60 column was used, but it was soon abandoned because of inadequate flow rate. Therefore, Biogel P6 was used in these columns from which it has been possible to obtain excluded peaks which have intermediate transfer activity. Unfortunately, they also have succinic thiokinase activity, but this was to be expected. Data from a successful experiment are shown in Table III. The protein concentration was 1.13 mg/ml as now determined routinely by 280/260 absorption measurements. In a similar experiment it was found that atractyloside, a gift from Dr. Robert Harris*, did not inhibit either succinic thiokinase or intermediate transfer activity to any appreciable extent. Further work at higher concentrations will be necessary, but these results do not agree with observations of others on atractyloside inhibition of succinic thiokinase from other sources. It was somewhat encouraging to find that the intermediate transfer activity was not inhibited.

*Univ. of Wis., Madison (contact made at Gordon Research Conference in August, 1968).

An effort was made to use columns of Biogel P6 equilibrated with double-distilled water rather than with buffer. If successful this would allow the excluded protein to be freeze-dried directly and reconstituted to desired concentrations. There was some encouragement that the water column could be used successfully, but the resulting excluded protein was still unstable and didn't tolerate freeze-drying or prolonged high speed centrifugation very well.

The most recent experiment employing acetone powder extract has been the most successful in some time. One of the reasons for this was that continued efforts to improve the reliability of the isobutanol-benzene extraction procedure have met with apparent success. The thorough mixing of one micromole of carrier phosphate with the sample before the addition of molybdate reagent significantly improved the precision of the data (one experiment). The net effect was more thorough and uniform extraction of $^{32}$phosphomolybdate as shown in Table IV. It was anticipated that gas chromatography in conjunction with radioactive labeling might be used to identify and quantitate the G-6-$^{32}$P produced by the intermediate transfer reaction. The normal amount of glucose in the assay system was excessive for gas chromatography, and the experiment was performed to determine whether a ten-fold reduction in the glucose level in the assay could be tolerated. As trimethylsilyl derivatives, glucose can be separated well from G-6-P on a gas chromatography column, but it was feared that the high levels of glucose in the samples might cause trouble due to overloading. A second objective was to confirm the stimulating effect of coenzyme A (CoA) upon the intermediate transfer activity. This experiment was the initial test of this stimulation in the presence of exogenous $NAD^+$. As can be seen in the results of

Table IV, there was no loss of activity by using ten-fold less glucose (still a saturating amount of glucose for hexokinase), and CoA did stimulate the transfer reaction significantly even in the presence of $NAD^+$. Perhaps further controls need to be run to make a very positive statement about CoA stimulation, but the gas chromatography results discussed below strengthen the case which is further bolstered by results obtained during the author's original investigation.

A few samples in this experiment were stopped by freezing rather than by the addition of trichloroacetic acid (TCA), so that they could be freeze-dried and submitted to gas chromatography after formation of trimethylsilyl derivatives. Although the amount of G-6-$^{32}$P formed in these reactions might be too minute to be detected by thermal conductivity in the gas chromatograph, it was planned to add carrier G-6-P in sufficient quantity to be detected easily and then to show that, among the fractions collected from the gas chromatograph, there was a $^{32}$P peak of radioactivity in exactly the same location in which the G-6-P peak emerged. The samples stopped by freezing were: (a) incubated with extract, (b) incubated with extract plus CoA, (c) zero time plus CoA without extract, and (d) incubated plus CoA without extract. Meanwhile preliminary work had been completed using the gas chromatograph to determine the behavior of knowns that would be present in a sample. No interference of other compounds, including extract protein, in the G-6-P region of the chromatogram was detectable at maximum concentrations expected in a sample. Problems did develop with bleeding of previously injected samples from the column during the running of subsequent samples. Thus, when the trimethylsilyl derivative of $^{32}$Pi was injected into the column, it was not completely recovered in a convenient peak but tended to appear in the column effluent as a burst followed by a long drawn-out tailing. There-

fore, the problem was (and is) to clean up the tailing or at least have it under control in a predictable way before introducing the next sample. A flushing procedure after each sample using cold carriers has been promising in this regard and has been used successfully in a limited number of trials. Efforts are being made currently to pack a new column with high performance stable packing material which may alleviate the tailing problem. There seems to be little question at this point that the gas chromatograph can be used successfully in a qualitative manner here. The quantitative analysis conceivably could require another method, perhaps thin layer chromatography.

It was found (Figure V) that indeed there was a peak of $^{32}P$ activity which coincided with the emergence of G-6-P from the chromatograph at 16-18 min. when samples (a) and (b) above were derivatized and injected. This was not the case when samples (c) and (d) were derivatized and injected. The data are preliminary but there is definite encouragement that G-6-P can be proven to be the product of the intermediate transfer reaction coupled with hexokinase. The importance of this proof was discussed earlier in conjunction with an attempt to accomplish the same task by paper chromatography (see pages 5-6).

It should be noted that, in addition to what is thought to be a permanent improvement in the isobutanol-benzene extraction procedure, much of the counting is now conducted without cocktail, using a new procedure based upon Čerenkov radiation produced by the energetic $^{32}P$. Since $^{14}C$ does not emit hard enough beta particles to give Čerenkov radiation, cocktail is still used for $^{14}C$ and other weak isotopes.

Outlook

The direction of the research in the immediate future is toward a

conclusive demonstration that G-6-P does result from coupling the intermediate transfer reaction to the hexokinase reaction as shown below:

$$(1)\ A\sim X + {}^{32}Pi \rightleftharpoons X\sim{}^{32}P + A$$

$$(2)\ X\sim{}^{32}P + ADP \rightleftharpoons AT^{32}P + X$$

$$(3)\ AT^{32}P + Glucose \xrightleftharpoons{\text{hexokinase}} G\text{-}6\text{-}{}^{32}P + ADP$$

This will be done qualitatively and, hopefully, quantitatively by gas chromatography. Preliminary results reported above indicate that G-6-P is the product and that an effort must be made now to obtain convincing publishable data. Besides the formation of G-6-$^{32}$P from $^{32}$Pi, $^{14}$C Glucose is also available and will be used as a further check that glucose is converted to G-6-P. Furthermore, $^{14}$C G-6-P is on hand and can be used to monitor any glucose-6-phosphatase activity occurring during the incubation. The phosphorylation of the 6 position of glucose, catalyzed by hexokinase, requires the energetic compound, ATP, rather than Pi whose reaction with glucose is energetically unfavored. The formation of AT$^{32}$P from ADP and $^{32}$Pi requires the input of at least 8,000 calories. The logical sources of such energy in a mitochondrial extract (presuming contaminants are not responsible as discussed earlier) are the "hypothetical" high energy intermediates of oxidative phosphorylation (A∼X or X∼P above) or high energy intermediates of the substrate phosphorylation mediated by succinic thiokinase which catalyzes the reaction shown below:

$$\text{Succinyl CoA} + ADP + Pi \rightleftharpoons \text{Succinate} + CoA + ATP$$

This is an overall reaction which does not show intermediates but rather the use of energy from succinyl CoA for the formation of ATP. Note that CoA is a normal component of this reaction, a fact which makes the stimulation of the

intermediate transfer reaction by CoA in the absence of exogenous succinate difficult to establish because of the possibility of cross reaction with the succinic thiokinase system.

In addition to product identification, further effort will be made to resolve the nature of the CoA stimulation of the intermediate transfer reaction, stimulation which is independent of exogenous succinate. There are three new tools on hand now that may make this resolution possible short of separation of succinic thiokinase and intermediate transfer activities, an ever-present goal. These tools are: $^{14}C$ labeled succinic acid; panthethine, an oxidized form of pantetheine which has been found to substitute for CoA in true succinic thiokinase activity from other sources; atractyloside which, in higher concentrations, may still prove useful.

These two major investigations on the G-6-P product and the CoA effect will occupy at least the next few months of work on this project. If firm data for the G-6-P product and substantial diagnostic evidence can be obtained demonstrating that there is a CoA effect on the intermediate transfer reaction which is distinct from its role in succinic thiokinase, I think a publication would be in order in late spring. This would probably be a short communication, perhaps initially to Bioenergetics Bulletin, to be followed by a full-length paper when more data on this and other aspects of the project are accumulated. If the initial work is completed in time, it might also be presented at the International Botanical Congress this summer.

There would not seem to be any unusual expenses in the work projected for the next few months. It might become necessary to try another technique

like thin layer chromatography, but the cost there would not be extraordinary, particularly if facilities and equipment located elsewhere in the Institute would be available.

Table I

Stability of Intermediate Transfer Activity at 4°

Crude acetone powder extract - standard $^{32}P$ assay at $10^{-4}$ M orthophosphate

| Extract Age at 4° | $NAD^+$ | Pi Incorporation* millimicromoles/sample/40 min. |
|---|---|---|
| fresh | - | 0.41 |
| | + | 1.60 |
| 1 day | - | 0.04 |
| | + | 0.19 |
| 1 week | - | 0.00 |
| | + | 0.16 |

*triplicate averages

Table II

Analytical Disk Electrophoresis of Material Excluded from Biogel P6 after Incubation with $^{32}Pi$ in the Absence of ADP

| Treatment | $NAD^+$ | $MgCl_2$ | Pi Incorporation cpm/gel tube |
|---|---|---|---|
| Background | | | 57 |
| A | - | - | 360 |
| B | + | - | 268 |
| C | + | + | 245 |
| D | - | + | 263 |
| E | - | - | not counted |

Table III

Intermediate Transfer Activity of Protein Excluded from Biogel P6
Standard $^{32}P$ Assay at $10^{-4}$ M Orthophosphate

| Time (min.) | Pi Incorporation* cpm/ml sample aliquot |
|---|---|
| 0 | 936 |
| 10 | 1292 |
| 20 | 1446 |
| 30 | 1309 |
| 60 | 1517 |
| Succinic Thiokinase Activity | |
| 0 | 752 |
| 60 | 109217 |

*triplicate averages

Table IV

Intermediate Transfer Activity of Protein Excluded from Biogel P6
Standard $^{32}P$ Assay at $10^{-4}$ M Orthophosphate

| Time (min.) | Glucose Concentration | CoA | Pi Incorporation* cpm/ml sample aliquot | |
|---|---|---|---|---|
| | | | Without cold Pi | With cold Pi |
| 0 | $2.5 \times 10^{-2}$ M | - | 2982,5851,4016 | 831,699,705 |
| | | + | 3523,6880,5864 | 930,771,846 |
| 20 | | - | 1899,2319,2349 | 1077,1095,933 |
| | | + | 2698,3375,2497 | 1365,1317,1377 |
| 0 | $2.5 \times 10^{-3}$ M | - | 3260,2815,2418 | 735,753,714 |
| | | + | 10359,2333,4238 | 735,726,723 |
| 20 | | - | 2056,4116,3096 | 1077,1083,1002 |
| | | + | 3023,3367,7883 | 1467,1410,1428 |

*triplicates

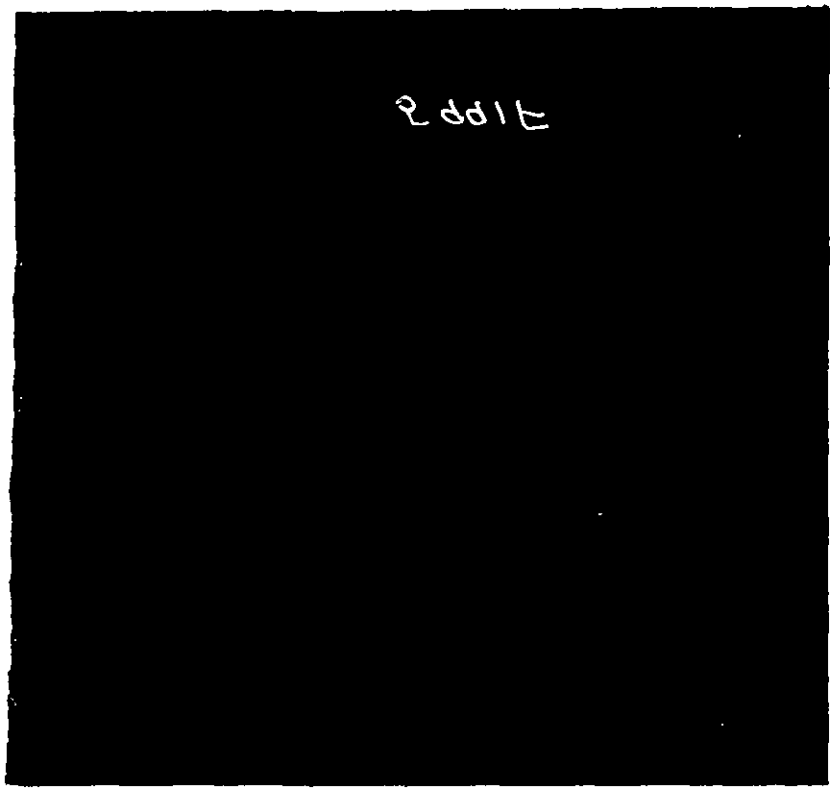


Plate I

"P (a) and "light" (b) cabbage mitochondria. Magnification
. Most of these mitochondria are slightly less than one
n "diameter."


Plate II

Cabbage mitochondria. Magnification 3,000 x. This micrograph at low magnification shows the degree of contamination of a typical preparation.

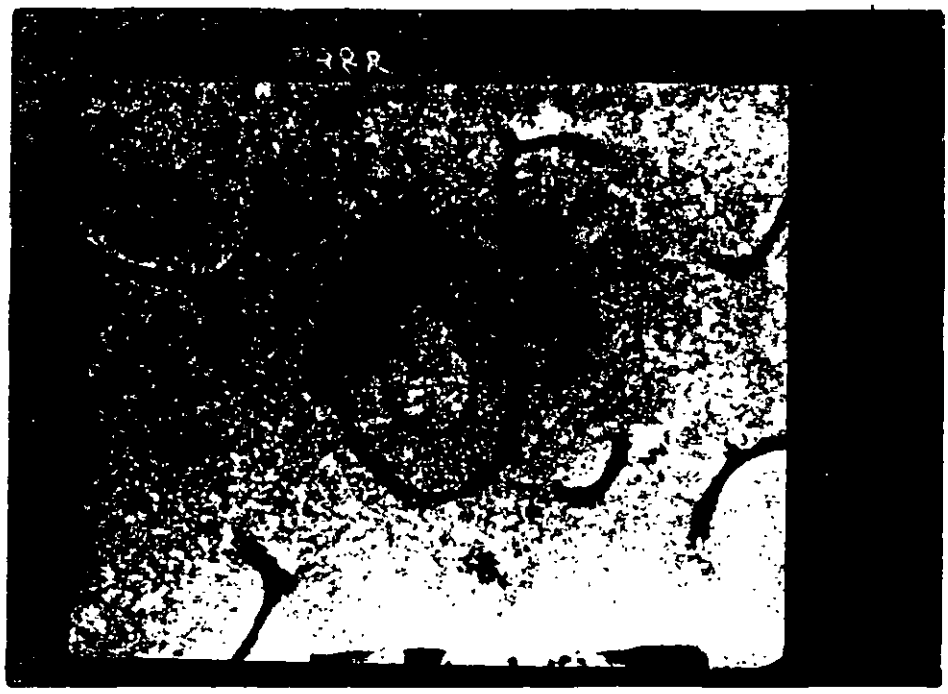


Plate III

Material sedimented from a crude extract of cabbage mitochondrial acetone powder in 14 hours at 105,000 x. g. Magnification 10,700 x.

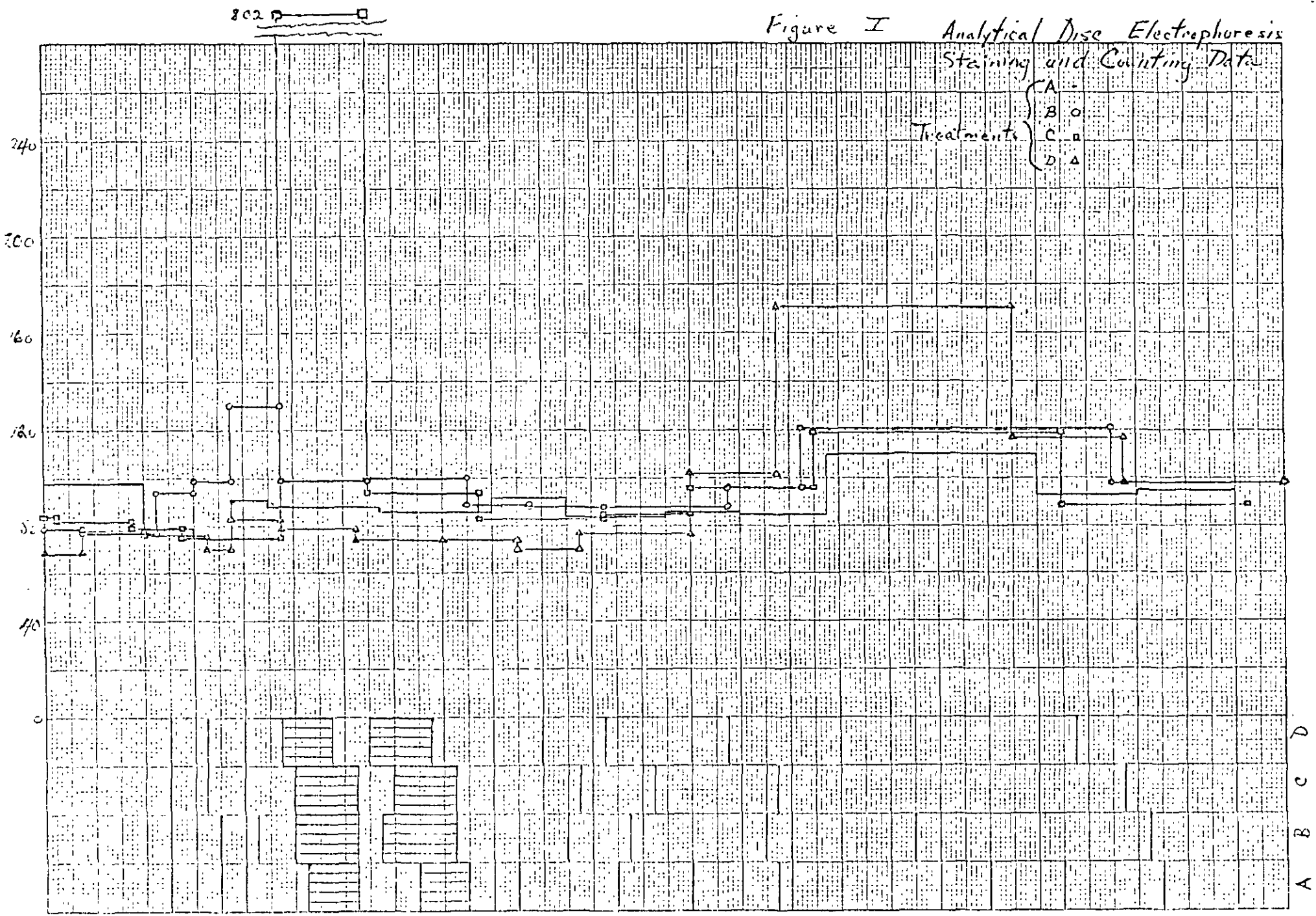



Figure I Analytical Disc Electrophoresis Staining and Counting Data

Figure II

Fraction E - Biogel P6 Elution

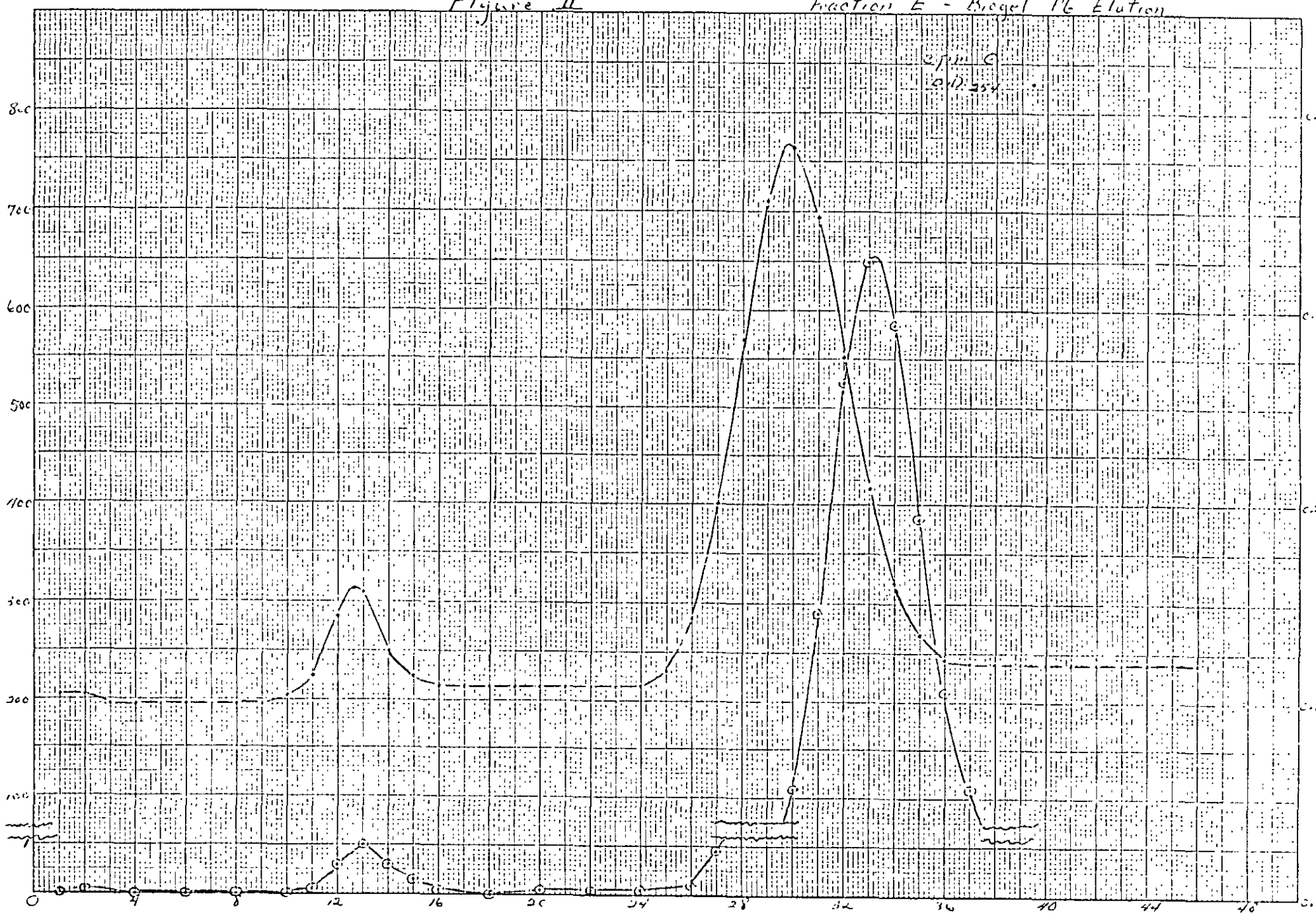


Figure III

Fraction E Included Peak - Biogel P2 Elution

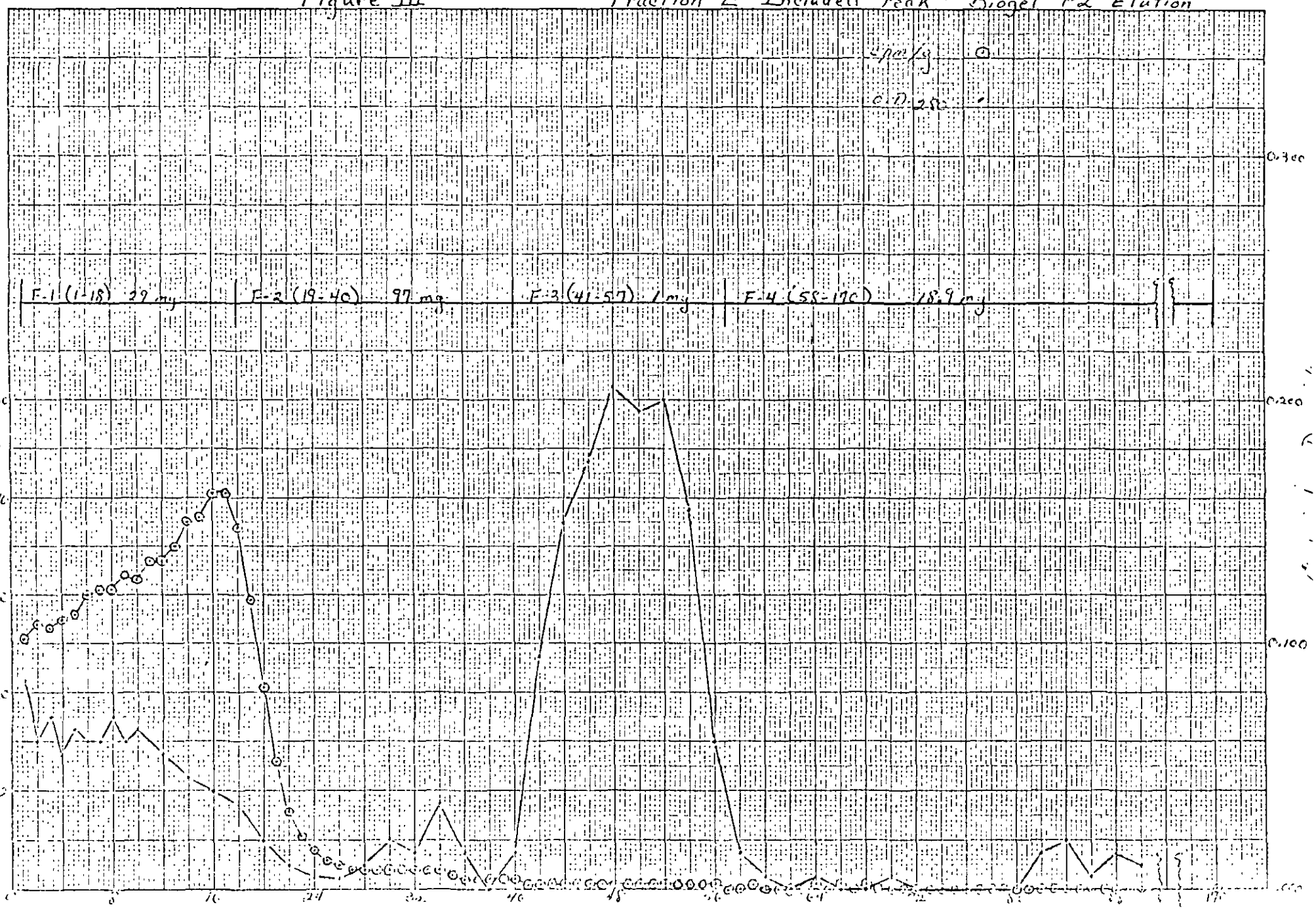


Figure IV

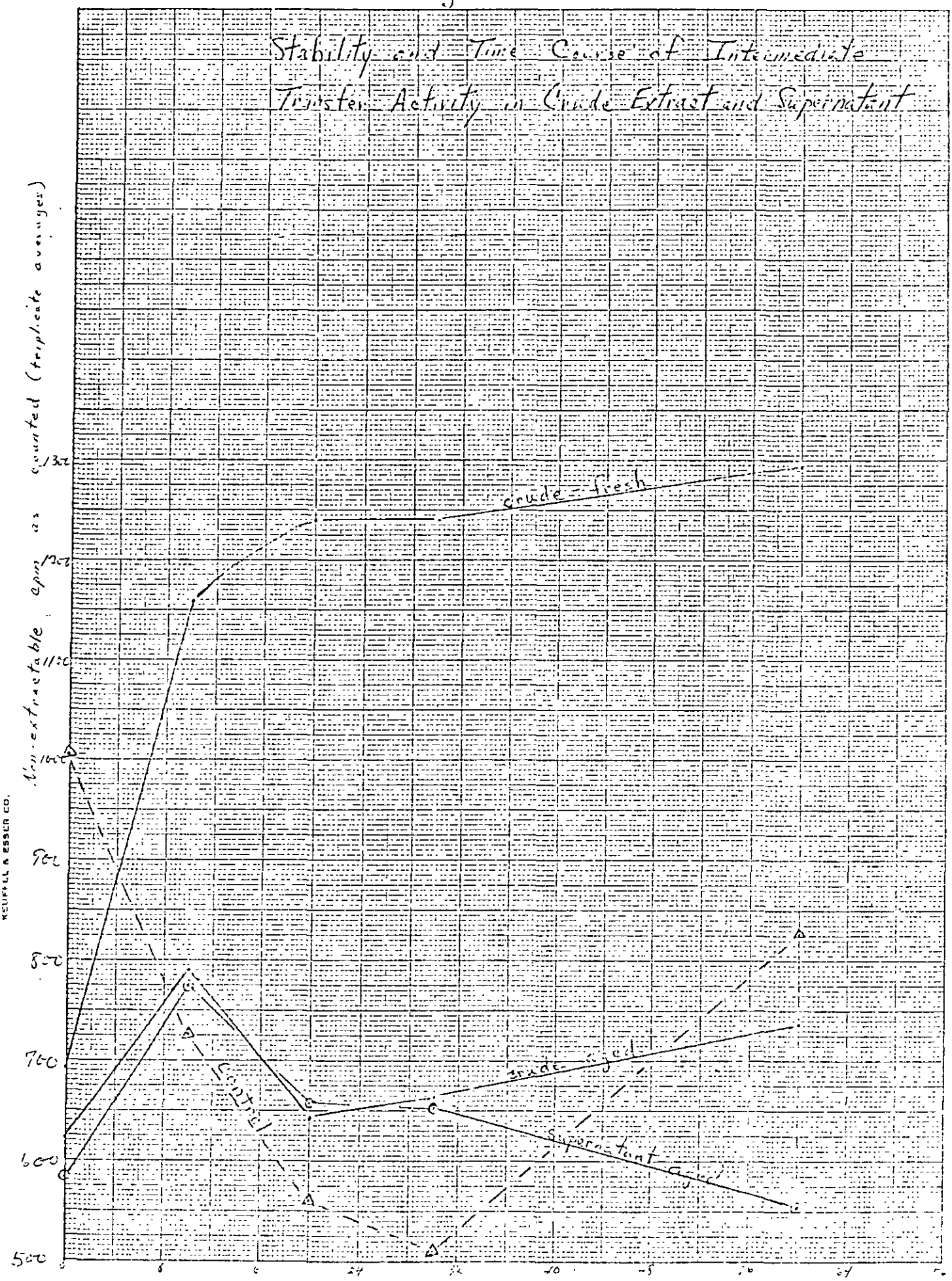
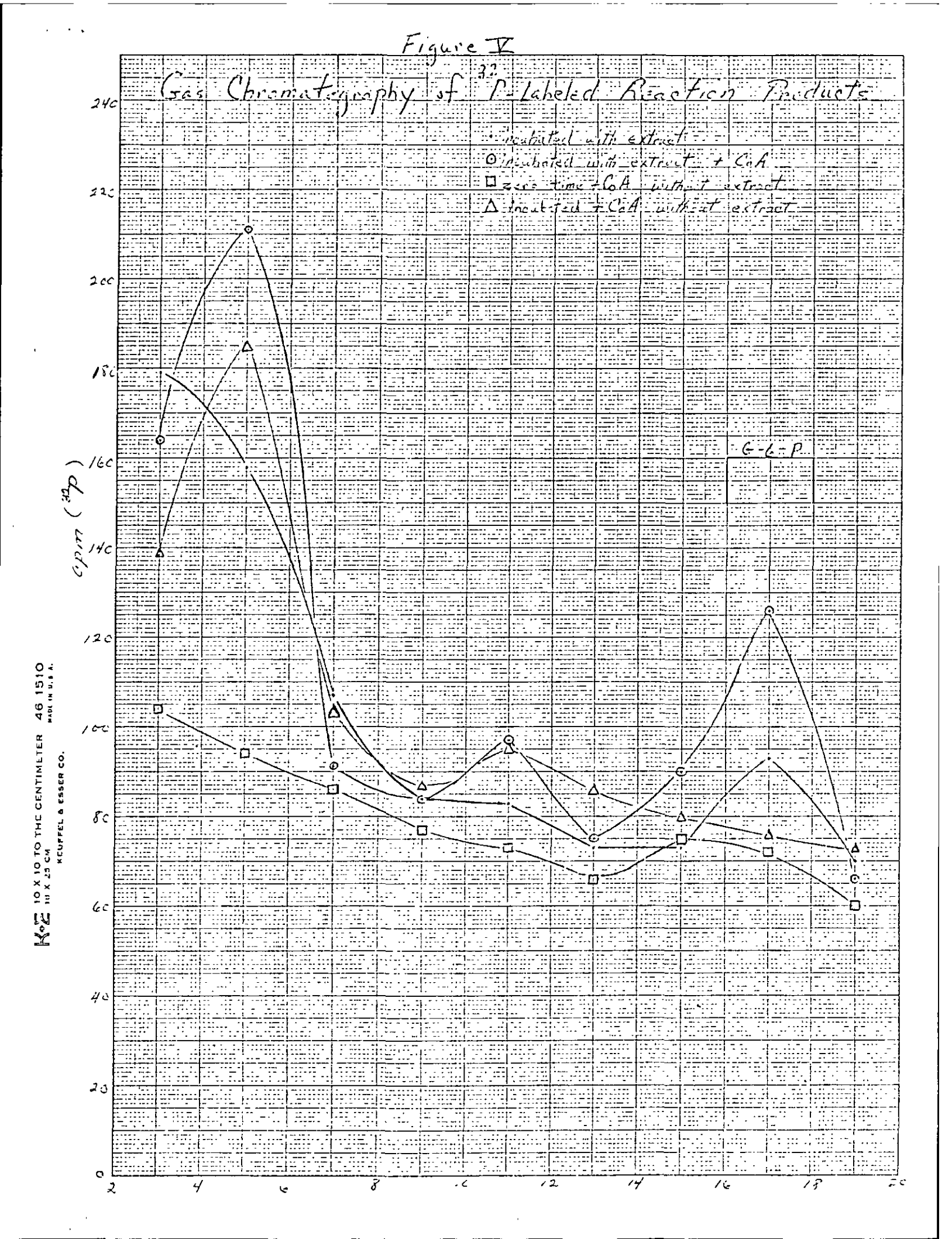

Stability and Time Course of Intermediate Transfer Activity in Crude Extract and Supernatant
counted (triplicate averages)
cpm
Non-extractable
1300
1100
900
700
500
300
crude - fresh
crude - aged
Control
Supernatant aged
0
6
12
24
32
40
48
56
64

Figure V


Gas Chromatography of 32P-Labeled Reaction Products
Incubated with extract
Incubated with extract + CoA
zero time + CoA without extract
Incubated + CoA without extract
G-6-P
cpm (32P)
240
220
200
180
160
140
120
100
80
60
40
20
0
2
4
6
8
10
12
14
16
18
20

# PROJECT REPORT FORM

PROJECT NO. 2691
COOPERATOR Institute of Paper Chem.
REPORT NO. 3
DATE April 27, 1970
NOTE BOOK 2620 pp. 144-160 and 2743
PAGE 1 TO 137
SIGNED Morris A. Johnson
Morris A. Johnson

Copies to: Files
Swanson
Einspahr
M. A. Johnson (3)
Carlson
Weiner

MECHANISMS IN BIOENERGETICS OF PLANTS


## Summary

Evidence which has accumulated over the period since the last report indicates that the soluble intermediate of oxidative phosphorylation isolated from cabbage mitochondria is of a ribonucleoprotein nature. Experiments are described herein, especially those involving inhibition of G-6-$^{32}$P formation from $^{32}$Pi by ribonuclease and fluorescent light, which lead to this conclusion. Other results point to the likely existence of a phosphorylated form of the intermediate and help to further our understanding of the role played by certain coenzymes and substrates when added to the extract. Evaluation of old and new assay procedures has led to increased confidence in the data obtained.


## Introduction

When the future direction of this project was prognosticated at the conclusion of the second report, it was hoped, among other objectives, that the new product analysis method described therein would allow conclusive demonstration that glucose-6-phosphate (G-6-P) was the product routinely measured by the old extraction procedure. Sufficient data was obtained on this point in the ensuing months to allow presentation of a paper at the XI International Botanical Congress in Seattle, September, 1969 (1). It was found that the extraction procedure used routinely in the past measured not only G-6-P but other unknown substances as well under certain conditions.

Armed with the new analysis specific for G-6-P, a variety of experiments were conducted early in this project report period attempting to assess the contributions of substrate phosphorylations and other reactions

to the capacity of mitochondrial extract to make G-6-P with the aid of hexokinase. Since November 1969, attention has been turned toward the possible involvement of ribonucleic acid (RNA) in the observed phosphorylation of glucose. Certain puzzling observations on the crude mitochondrial extract, such as its great instability and unusual absorption spectrum, coupled with observations in the literature that were reinforced by the experiments of others reported at the Seattle Congress, made it attractive to test an hypothesis on RNA involvement that the author had held for years but had previously considered too "wild" to warrant immediate investigation.

Because of previous reports in the literature (2,3) and the advantages of specificity involved, the simplest test seemed to be to find out whether the transfer reaction leading to G-6-P formation was sensitive to ribonuclease (RNase). All experiments to date indicate that pancreatic RNase does inhibit transfer activity; furthermore, an old suspicion that the observed lability of the transfer activity might be due to photoinactivation has also been confirmed, an observation which is also consistent with RNA involvement. Numerous other experiments are reported here relative to the mechanism of RNA involvement in oxidative phosphorylation; many of these observations require much further work to insure their reproducibility.

At the moment the most important findings are destruction of transfer activity by common laboratory fluorescent lighting and inhibition by RNase. The idea of RNA involvement in oxidative phosphorylation is not expected to be readily accepted by other workers in the field. The occurrence of RNA in mitochondria has been noted in recent years by several workers (4); however, in the light of the present stage of biochemical research there

are more apparent possibilities for its function than energy transduction. At least three kinds of RNA are well-known which have other established functions. Our experiments to date on RNA involvement may not in themselves exclude all other possibilities; the author is well aware of the possible necessity to satisfy himself as well as others on some of these points in the future, but the initial results are thought to be firmly grounded.

## Results and Discussion

### PART I

Because the old extraction method for G-6-$^{32}$P determination, while unspecific, is quite "sensitive" relative to the new gas chromatographic method, the first attempts to correlate the results of the two analytical procedures encountered some difficulty. The use of the term "sensitive" here is probably misleading because the lack of "sensitivity" of the gas chromatographic method is due principally to two operations preliminary to injecting the sample: (a) under the compromise conditions for silylation of reaction products, the yield of G-6-P silylated derivative is less than 20% and (b) it is only feasible to inject about 1/20 of the total volume in which the derivative is made. Thus, the "sensitivity" of the extraction procedure is greater due to the fact that one can look at a larger aliquot of the total sample. To illustrate this point, in experiment No. 18 (Figure 1) it was possible to detect nonextractable $^{32}$P in the presence and absence of coenzyme A (CoA) stimulation; however, when the samples were assayed by gas chromatography only the CoA-stimulated G-6-$^{32}$P formation was barely detectable. Experiment No. 19 showed that the lack of detection in the unstimulated (with CoA) case in No. 18 was probably just a matter of "sensitivity" (Figure 2 and Table 1). The peculiar break in the unstimulated time progress curve between 20 and 30 minutes in experiment No. 18 (Figure 1) has

not been investigated further; it did not occur in experiment No. 19 but the behavior of the nonenzymic controls (Figure 2 and Table 1) indicates the possibility that the blip may have been real but not G-6-$^{32}$P. From experiment No. 19 it became clear that, in the absence of extract, nonenzymic reactions leading to the formation of nonextractable $^{32}$P do occur, but this nonextractable $^{32}$P is not G-6-$^{32}$P; furthermore, the precise quantitative contribution of such unidentified labeled nonextractable material to the total labeled nonextractable $^{32}$P in the presence of extract is not easily assessed (note that the CoA-stimulated reaction does not "take off" again--Figure 2). It is clear that at least part of the nonextractable $^{32}$P, formed in the presence of extract only--not in the nonenzymic controls, is G-6-$^{32}$P and it is formed in a rapid reaction relative to the unidentified $^{32}$P-labeled material under the assay conditions. The stimulation of nonextractable $^{32}$P by CoA (and also by $NAD^+$ as shown later) is attributable to increased G-6-$^{32}$P formation. Assays by the gas chromatographic procedure are not encumbered by these uncertainties. As far as has been determined, no other component of the incubation mixture peaks in the region of the same retention time as G-6-P. A known incubation mixture yields peaks, but of lower retention time. A more rigorous examination of the homogeneity of the G-6-P peak region may be warranted in the future. At present it would seem that any possible interference would have to come from a compound of unknown nature; thus the specificity of the method is reasonably well-assured. It was shown that $^{14}$C G-6-P chromatographs with this peak although some breakdown to $^{14}$C glucose occurred. This degradation may have occurred in storage, in the gas chromatograph, or during derivatization. If operational, this degradation is probably a constant but should be investigated further with fresh $^{14}$C G-6-P (see discussion of G-6-Pase below).

On the basis of the foregoing and several subsequent experiments, it should be safe to state that the gas chromatographic method measures G-6-$^{32}$P and only G-6-$^{32}$P after a correction for the tailing of $^{32}$Pi in the baseline has been made. Nevertheless, the nonextractable $^{32}$P procedure has not been abandoned entirely. Its greater "sensitivity" and its capacity to measure other labeled organic molecules make it a good method to hold in reserve. For example, if the RNA hypothesis is correct, this procedure could measure $^{32}$P-labeled RNA (from past experience not discussed here, it is thought that this might be occurring).

It has been mentioned that known $^{14}$C G-6-P may undergo some degradation on gas chromatographic analysis. If such breakdown of G-6-$^{32}$P occurs, it wouldn't be detected because glucose would then be unlabeled. Because the $^{14}$C G-6-P known used in this work had been stored for some time and appeared to have picked up moisture, it cannot be ruled out that the degradation had occurred in storage. Attempts to assay G-6-Pase activity of the mitochondrial extract have been hampered by this nonenzymic breakdown of G-6-P, and no results will be presented since it is considered to be unreliable data at this point. This work should eventually be repeated with a fresh $^{14}$C G-6-P known mixed with a considerable amount of carrier G-6-P.

There was another portion of experiment No. 19 which yielded results which certainly deserve further investigation in the future also. In addition to samples in which the radioactive label was provided by the addition of $^{32}$Pi, there were also some in which the Pi was unlabeled while glucose was $^{14}$C-labeled and some in which both $^{14}$C glucose and $^{32}$Pi were present during incubation. While there are some inexplicable aspects to

these results, the general observation is that a large amount of glucose is converted to G-6-P which does not acquire the phosphorous from Pi (Table 2). This immediately poses at least three important possibilities: (a) there are considerable amounts of unlabeled ATP in the mitochondrial extract, (b) there is a phosphorylated intermediate ($X\sim\overset{\ddagger}{P}$) of oxidative phosphorylation in the mitochondrial extract or (c) the hexokinase used contains considerable amounts of ATP. Since nonenzymic controls usually at most show no more G-6-$^{32}$P formation than might be expected due to residual contamination from a previous sample, possibility (c) seems very unlikely. If (a) were correct, then this ATP must be in some kind of bound form or extracts excluded from Biogel P-6 should be completely inactive (not the case). Possibility (b) is, of course, attractive and would be consistent with the classical hypothesis of the oxidative phosphorylation mechanism. Since it would have an effect on the kinetics of labeling from $^{32}$Pi similar to the addition of excess carrier G-6-P to an incubation system (discussed later in this report), it may be useful in explaining apparent reversal of the G-6-$^{32}$P formation with time. Since some insight into these problems should be accessible experimentally, they will be investigated as soon as time and priorities permit. The glucose concentration was slightly higher where $^{14}$Cglucose was used - (3.12 versus 2.5 mM), but it is extremely doubtful that this played any significant role in the results of Table 2.

In experiments No. 20 - 26, the major concern was with the extent to which substrate phosphorylations were contributing to the observed results, especially as now being obtained with the specific gas chromatographic procedure. From the results of experiment No. 20 (Table 3) it was apparent (within the limitations of the experimental setup) that both $NAD^+$

$\ddagger$ for this notation see report No. 2, p. 14.

and G-3-P stimulated G-6-$^{32}$P formation from $^{32}$Pi with $NAD^+$ being more potent in this regard. When used together, the stimulation was far more than additive. The results continued to give the impression that, due to rapid reaction, a zero time could not be obtained. The small figures in the nonenzymic control can logically be ascribed to contamination since these two samples were run after the highest count sample. At first these results were quite shaking for it appeared that some combination of glyceraldehyde-3-phosphate dehydrogenase and phosphoglyceric kinase (two glycolytic or chloroplast enzymes) contaminating the cabbage mitochondrial preparation might account for the results observed to that date. However, there were still questions (among others) of the nature of the unstimulated activity, whether $NAD^+$ stimulation of this unstimulated activity (if not a contaminant) might be distinct from its behavior when added with G-3-P, and of whether G-3-P was undergoing oxidation in the process of promoting G-6-$^{32}$P formation from $^{32}$Pi. Since the glycolytic enzymes, if present, should be adsorbed to particles in the mitochondrial preparation (not the mitochondrial extract) and subsequently extractable from the acetone powder, two experiments (No. 21 and 25 - Table 4) were run to see whether increasing the washing of mitochondria (normally washed twice) might reduce the G-3-P and/or $NAD^+$ stimulation of $^{32}$Pi incorporation; it was hoped that it might be possible to destroy (wash off) the stimulation by G-3-P, for example, while leaving unstimulated and/or $NAD^+$-stimulated activity. CoA was left out at this point in an attempt to keep an already complicated system from becoming more so. The results of experiment No. 21 were somewhat encouraging and at the same time rather surprising in that washing <u>increased</u> the $NAD^+$ stimulation. At the same time the G-3-P stimulation was static indicating, along with the fact that the unstimulated reaction was increased

by washing, that perhaps the $NAD^+$ was participating in another reaction that did not involve G-3-P. In experiment No. 25, which was a more elaborate version of experiment No. 21, it appeared that any involvement of exogenous G-3-P was abolished in 4x or more washed mitochondria while unstimulated and $NAD^+$-stimulated activity remained. In both of these experiments there were some differences which were altered slightly depending on whether or not the results were expressed on a per mg protein basis. It might be fruitful to wash more than 8 times (note $NAD^+$ stimulation appears to be declining yet after 8 washes), but it promises to be a monstrous undertaking; furthermore, this much washing and the time element involved may be leading to leaching and degradation of mitochondria as opposed to deadsorption.

Experiments No. 22 and No. 24 were attempts to attack particulate (chloroplast-related) contamination problems by employing density gradient centrifugation techniques on the mitochondrial preparations. The general result of these investigations was that with our present equipment, it is not feasible to use gradient centrifugation as a preparative procedure, although it might well be used analytically on a small scale. Experiment No. 23 was an attempt to prepare white potato mitochondria and use them as a source of mitochondrial extract in hopes that a system free of green chloroplasts might be devoid of some contamination problems. In this "one-shot" attempt, it was quite a gamble to expect to produce good mitochondria in this system without considerably more effort and, somewhat as expected, it failed and has not been pursued further.

In the last experiment run (No. 26 - Figure 3) before launching the investigation into RNA involvement, the effect of reduced $NAD^+$ (NADH)

upon the unstimulated versus the G-3-P plus $NAD^+$-stimulated transfer reaction was investigated. NADH should inhibit glyceraldehyde-3-phosphate dehydrogenase. If the unstimulated reaction were of the same nature, the same percentage inhibition might be expected there also. Two levels of NADH were employed, one below and one above the $NAD^+$ concentration used. The results can be interpreted as follows, although other possibilities may exist: the unstimulated activity was inhibited about 50% by NADH at 100 μM but no additional effect was forthcoming at 1 mM. Meanwhile the G-3-P plus $NAD^+$ complete system was inhibited about 80% by 100 μM NADH and 90% by 1 mM NADH. The upturn in 5 to 10 min. at the 100 μM NADH level (also may be evident in the unstimulated case) is probably due to the fact that NADH undergoes autooxidation (chemical) in solution which both raises the level of $NAD^+$ (a stimulant) and reduces the level of NADH (an inhibitor). The same amount of oxidation occurring in the solution where the initial NADH concentration was 1 mM would still leave a preponderance of NADH, the inhibitor, so no upturn is seen. Since there was no shortage of G-3-P (3.68 mM), it really doesn't seem that the glyceraldehyde-3-P dehydrogenase system is involved or 1 mM NADH inhibition should have been released also.

For the most part, $NAD^+$ and CoA were not employed in the experiments of Part II of this report. This is not meant to imply that experimentation with these coenzymes has been abandoned but only that the possibility of RNA involvement is a matter of first magnitude importance. Since the work in Part II has been under way, it has become evident that some of the problems pursued here in Part I may now be investigated more fruitfully employing such criteria as susceptibility to RNase and light. The results of the washing experiments and the NADH experiment imply that the $NAD^+$

stimulation is closely linked to the unstimulated system and, if it is the result of contamination which seems less and less likely as more experiments are done, the contaminant must be particulate (e.g., from immature chloroplasts) in the mitochondrial preparation.

PART II

As noted on page 10 of the last report, protein determination is conducted now routinely by use of the 280 nm/260 nm method of Warburg and Christian (5). The longer this procedure was used, the more striking became the reproducibility of this ratio which for crude extract has been 0.75 $\pm$ 0.01 in 10 of the last 12 preparations; the other two preparations had ratios of 0.79 and 0.70. For active preparations excluded from Biogel P-6 (i.e., low molecular weight substances removed) the ratio has been somewhat higher, 0.84 to 1.01. Pure protein preparations have 280/260's of 1.75 or higher (some above 2.00), while pure yeast nucleic acid has a ratio of 0.49, thus, the common reaction to a ratio of 0.75 would be that the preparation is contaminated considerably with nucleic acids. However, it doesn't really seem reasonable that a contaminant should appear in such a consistently reproducible quantitative relationship considering that this extract comes from a biological source where reproducibility is more often the exception than the rule. Therefore, the position has been taken that polymeric material responsible for 260 nm absorption in the extract is not a contaminant but exists bound to protein in vivo. Since the most obvious biopolymer of this type is nucleic acid, it is proposed, considering further observations presented below, that a nucleoprotein is being isolated which acts like or is an intermediate in oxidative phosphorylation. A scan (Figure 4-a) of the visible and ultraviolet (U.V.) spectrum of a recent

extract showed that the only peak was in the 260 nm region in agreement with previous scans on extracts in the past.

It was known (see report 2, p. 3) that the sedimentation pattern of crude extract in the ultracentrifuge usually consisted of only two peaks of which the one at about 4S was by far the major. The other very small peak in the 7S region (sometimes two very small peaks are seen) was apparently the highest molecular weight material in the preparation. The only kind of nucleic acid that would be expected to have sedimentation coefficients this low is soluble (transfer) RNA or some other RNA's of this type with no function currently assigned to them. With this in mind, it was decided that the first thing to try was to find out whether incubation of extract with RNase (enzyme hydrolytically cleaving RNA) would have any effect on the sedimentation profile. In the first attempt to demonstrate destruction of a sedimentation peak it soon became obvious that the fact that RNase is itself a very small protein which sediments around 2S would complicate interpretation of what was happening in the 4S region. Something destructive obviously took place when RNase was mixed with crude extract, for there was almost immediate visible denaturation.

Since there were going to be problems with the ultracentrifuge as a useful tool but visual observation indicated that RNase was reacting with extract, the next experiment (No. 28) provided the first test of RNase inhibition of G-6-$^{32}$P formation from $^{32}$Pi (Table 5-A). The effect of trypsin (a proteolytic enzyme) upon the transfer reaction was also studied although controls have not yet been run to find out if the trypsin inhibition observed is due to an attack on hexokinase. Both enzymes were inhibitory in this initial test and this has been confirmed in subsequent experiments (Table 5

also). The progress of the reaction can be seen in Figure 5, a rapid reaction followed by apparent decline in G-6-P yield. Among the data can be seen other variables of interest such as the effect of aging in the dark or in fluorescent light (4° or room temperature) upon subsequent activity and also tests of exogenous ADP dependence. One may note in Table 5 that the yield figures are considerably higher in experiments No. 28 and No. 32 than in No. 30-31. The major reason for this is not that less G-6-P was formed per unit time but that a modification in procedure resulted in obtainment of specific activity figures in No. 30-31 that were too high and progressively so at longer incubations. The deleterious effects of the modification, which is briefly described below, would be most significant for the study of exogenous ADP as an incubation variable while less significant for the study of other variables.

The modification mentioned above was essentially the addition of carrier G-6-P to the incubation medium rather than adding it later prior to gas chromatography. It was realized that such a modification would probably disadvantageously reduce yields, but, as long as significant cpm could be obtained, it held certain advantages such as better mixing of carrier with label and reducing the number of times a set of samples had to be processed during analysis. With hindsight there was one more disadvantage which dictated abandonment of the modification, namely, a dilution of specific activity of $AT^{32}P$ occurs during incubation. While the hexokinase reaction below (keq $\approx$2500 in favor of G-6-P) is generally classified as an "irreversible" biochemical reaction, it can be reversed (6) by loading the right-hand side, as is done when carrier G-6-P is added to the incubation medium.

$$\text{Glucose} + \text{ATP} \overset{\text{hexokinase}}{\rightleftharpoons} \text{G-6-P} + \text{ADP}$$

If ADP is also supplied, there is opportunity to form ATP (unlabeled) and dilute the specific activity of labeled $AT^{32}P$ arising from the reactions of the extract. As a result (increasingly so with time, especially if no exogenous ADP is provided initially) glucose is being phosphorylated by $AT^{32}P$ of lower specific activity than the specific activity of the $^{32}Pi$ used for calculations of G-6-P yield. Thus calculated yields under conditions where large amounts of G-6-P are present will be reduced by the contribution due to reversal of the hexokinase reaction. Experiments No. 28 and No. 32 are not complicated by this factor and permit some reasonable interpretation of the results of the intervening experiments. The decline in apparent G-6-P yield seen in Figure 5 is thought to be related to a similar situation in which the "cold" G-6-P originates from X~P.

At this time inhibition by RNase (two different commercial sources) and fluorescent light can be presented with confidence. However, meager results obtained by adding bovine serum albumin (BSA), a nonenzymic protein, in some of these experiments indicated that there is a remote possibility that inhibition by RNase is not due to hydrolytic cleavage of RNA. The purity of RNase, trypsin, or BSA used also must be considered; for example, it has been reported (7) that many commercial preparations of trypsin contain some RNase activity (vice versa is not too likely since RNase is a small protein, and one of the most investigated enzymes known; BSA is a question mark).

Some further effort has been expended attempting to correlate RNase attack with a change in ultracentrifuge pattern. However, while this has yielded interesting results, they are difficult to interpret because the nucleic acid is bound to protein (further evidence of this will be discussed),

and also because it is probable that very subtle changes (not easily detectable by physical methods) in RNA may be caused by RNase resulting in the loss of capacity to make ATP. Similar reservations apply to trypsin and will be mentioned again in connection with light sensitivity. From what has been observed of the RNase effect in conjunction with agents which disrupt non-covalent linkages such as urea, phenol, phosphate, sodium chloride and another reagent used to reduce disulfide bridges, dithiothreitol, a meaningful study with the analytical ultracentrifuge would be a project in itself and perhaps should await the availability of data from further chemical and physical characterization of the extract by other means. It would seem possible that the RNase may cleave RNA but leave all nucleotide fragments associated with protein. As a result, no change in sedimentation profile would be observed even though RNA had been cleaved. The use of deproteinized extract (described later) should help to resolve this problem. Certainly changes (some drastic) have been noted in sedimentation profile with the chemical agents described, in conjunction with RNase or alone, but no interpretation can be put on these results at this stage of the investigation. A synthetic boundary run on deproteinized extract has given us a tentative value of 1.5S for the RNA.

Exploratory work was conducted on the nature of the polymeric material responsible for 260 nm absorption and on what happens when RNase, trypsin and BSA are incubated with extract or deproteinized extract. This phase of the investigation utilized both crude mitochondrial extract and, in some cases, extract from which the protein had been removed by the Sevag procedure (this amounts to denaturation of the protein with chloroform and removal of the chloroform-protein gel that is formed).

After extract was incubated with RNase at room temperature for 30 hours, subsequent paper chromatography revealed a U.V. absorbing spot that had an $R_f$ compatible with its being an adenine nucleotide plus two spots which could be described as white fluorescent when examined with the U.V. lamp ($\lambda$ = 254 nm). A weaker U.V. absorbing spot at the same $R_f$ was seen following identical incubation with trypsin, but it was not as intense; incubation with BSA resulted in a very weak absorbance in this region. This substance was also present in an untreated control just slightly more intense than in the BSA case and much weaker than in the trypsin - or RNase - treated cases. Controls of RNase, BSA or trypsin alone did not show this material; aging untreated extract showed that it underwent autodigestion with time to increase the intensity of this spot. Therefore, a substance with mobility like an adenine nucleotide was liberated by RNase attack, and less so by other agents. BSA seemed to protect against deterioration of the extract leading to the same product. However, examination at two pH's of the U.V. spectrum of this product after elution with double-distilled water showed that, while adenine may be present in this material, the dominant absorption peak is at 249 nm at pH 1. A good lead to the nature of this material may lie in the observation that deaminated adenine nucleotides (including deaminated $NAD^+$) have absorption peaks in this region. The recovery and spectral examination of knowns that have been through the same procedure may prove to be very interesting. Ninhydrin-reactive chromatographic spots have also been detected resulting from aging or enzymic attack on extract. Only brief mention of the more interesting results will be made here. A major component of the extract which is not U.V. absorbent but ninhydrin positive appears to be a degradation product of a very labile protein. Because of the color given with ninhydrin (yellow) it is thought to be proline or a proline-related amino acid. In general, trypsin promotes the formation of this

material as well as spots with $R_f$'s like valine and lysine plus some yet unidentified amino acids. The presence of BSA seems to suppress both protein and nucleic acid degradation. It is well to mention at this point that there is some other unidentified material of low $R_f$ coming from the extract which does not absorb U.V. light nor give a color with ninhydrin (leaves a very white spot). Initially it was thought that this might be Pi since it gave a yellow spot with Wade-Morgan reagent (8), but later work revealed that Pi runs near the solvent front. Perhaps this material is lipid or carbohydrate but it remains unidentified at present.

Chemical hydrolysis of extract and deproteinized extract under various conditions has been useful in elucidating the nature of the 260 nm absorbing material. The first attempt in this direction was hydrolysis of crude extract in 1N HCl for one hour at 100°C (a standard hydrolysis regimen for RNA). Paper chromatography of the hydrolysis products revealed only two U.V. absorbent spots with $R_f$'s of about 0.83 and 0.95; of these two, the spot near the solvent front was the larger and some of the material seemed to run with the solvent and spread out along the front. Other materials could be present but too weak to detect. At least 25 µl and preferably 100 µl were needed to pick up the 0.83 and 0.95 $R_f$ spots. Unhydrolyzed control extract did not seem to show much U.V. absorption on the chromatogram at all, even though it is known to absorb U.V. around 260 nm; perhaps this is a reflection of the fact that extinction coefficients for nucleic acid monomers are greater than for polymers of the same composition. The U.V. absorption spectrum of the eluted 0.95 $R_f$ major spot was determined as is (about pH 4 - Figure 6) and at various other pH's; the 0.83 $R_f$ substance yielded similar but not identical results. It can be seen that, from the extract which has a $\lambda$ max

around 260 nm, the major constituent from acid hydrolysis yields a symmetrical absorption peak with λ max above 280 nm. From comparisons with known spectra in the literature, a derivative of cytidylic acid would seem to be a likely candidate but no precise correlations with known spectra have been obtained.

Extract has also been hydrolyzed in 72% perchloric acid for one hour at 100° (this is strong enough to hydrolyze both RNA and DNA). Definite U.V. absorbing spots were found at $R_f$'s 0.46 and 0.83. The 0.46 material traveled as one would expect of cytosine but its absorption spectra did not agree, showing peaks at 248 nm and 320 nm. The 0.83 $R_f$ here was too weak to get a good spectrum.

The removal of protein from the extract causes very little change in the absorption spectra (Figure 7-a). Hydrolyses of deproteinized extract have been conducted in 1N HCl at 100°C for one hour, in 1N HCl at 24°C for 100 hours, and in 1N KOH at 24°C for 18 hours, and in RNase solution at 24°C for 24 hours. Chromatographically hydrolyzed deproteinized extract seems to behave much the same as if hydrolyses were conducted with the protein present. Thus, hydrolyses in 1N HCl under both of the above listed conditions yielded only two U.V. absorbing spots of approximately the same $R_f$'s reported previously for whole extract (p. 16 this report). The same can probably be said for RNase hydrolysis; however, here as in KOH hydrolysis the spots were too weak to draw firm conclusions. In all cases where hydrolysis products of deproteinized extract were examined, higher initial concentrations are needed - no good U.V. spectra have been obtained using eluted spots. Nevertheless, the effect of RNase on the U.V. spectra of deproteinized extract has been determined (Figure 7) and would seem to be consistent

with the observations of others (9) on the results of RNase attack upon known RNA in that an initial small increase (later increases can be traced to controls) in absorption was seen at the λ max of deproteinized extract. An interesting sidelight developed in this work in that after 24 hours the λ max had shifted to about 245 nm. It was found by examining the control spectra (not shown here) that this shift was occurring in the absence of RNase. A possible explanation for this behavior may be that photochemical addition of water to a pyrimidine double bond is being witnessed (10). It may be tied to inactivation of transfer activity by fluorescent light noted earlier, and is certainly deserving of much further investigation.

A number of chemical tests have been run on deproteinized extract and some eluted chromatographic spots. Two colorimetric procedures were employed which are claimed to allow one to distinguish between RNA and DNA. The procedure of Hahn and von Euler (11) has provided evidence (Figure 8) that the deproteinized extract and the major high $R_f$ spot from acid hydrolysis are RNA and an RNA hydrolysis product respectively. As of this writing a standard curve with yeast RNA has not been run, but the λ max of color development with samples has been at 680 nm in agreement with the published procedure. An attempt to use a ribose standard did not work ideally since the color development peaked at 630 nm; yeast RNA known is on hand and will be run in the very near future. The procedure of S. S. Cohen (12) which is supposed to yield a DNA color with λ max at 500 nm and an RNA color with λ max 640-700 nm has not been successful because the color developed in our experience peaks at 580 nm. The more conventional procedures (using diphenylamine and orcinol reagents for DNA and RNA respectively) have yet to be tried. Since there is little indication that significant amounts of purines are present in this RNA, these procedures may not be too meaningful

in this case. The low molecular weight of this nucleic acid argues against its being DNA. A further confirmation may be obtained by comparing the effects of RNase and DNase attack (not yet tried).

A number of spot tests (both on a spot plate and on paper) have been run on the eluted chromatographic products from HCl and $HClO_4$ hydrolyses. Briefly, phosphorus tests on the 0.95 $R_f$ material indicate that the compound is phosphorylated. Ferrous and ferric iron (possible chelators of $PO_4^{\equiv}$) both seem to be present. Most color was obtained from a procedure in which $Fe^{+++}$ was reduced with $Na_2S_2O_4$ and then tested for $Fe^{++}$. Sulfur tests were also conducted based upon the CoA stimulation of transfer activity, the effect of dithiothreitol treatment on the ultracentrifuge profile, and the fact that the 0.46 $R_f$ spot from 72% $HClO_4$ hydrolysis absorbed in the 320 nm region. The test for sulfur has been negative to date but perhaps it is not sensitive enough since only very weak eluates of the 0.46 $R_f$ material have been available. Utilization of the sulfur test (Na $N_3$ in $I_2$-KI) in a spray reagent has been fruitful since it detects the location of the MOPS buffer in use and has raised the suspicion that the material previously mentioned as being lipid or carbohydrate may contain sulfur. Most spot tests were disadvantaged by the development of some color in blanks necessitating a judgment on differences over and above a blank.

Some work employing a commercial preparation of polynucleotide phosphorylase (PNPase) should be discussed. This enzyme catalyzes a non-specific phosphorolysis of RNA yielding nucleoside diphosphates. It occurred to the author that it might provide a new handle on the RNA composition if this phosphorolysis of deproteinized extract were to be run in the presence of $^{32}Pi$. This should result in the liberation of $^{32}P$-labeled nucleoside di-

phosphates that could be chromatographed with various knowns to aid in identification of small amounts of material that could not be detected otherwise. Because $^{32}Pi$ was found to run just behind the solvent front in paper chromatography, it was found necessary to go through the molybdate extraction of $^{32}Pi$ in the incubation mixture prior to chromatography. While this has its complications, the results are extremely interesting. The results of two such experiments agree and are very intriguing as can be seen in the results of the second run (Figure 9). Assuming that the products of PNPase phosphorolysis are chromatographically similar to those of HCl hydrolysis, one could suggest that there is considerable label in regions which would roughly correspond to the unidentified substances of $R_f$ 0.95 resulting from HCl hydrolysis. Label also occurs in other unidentified peaks while small amounts of labeled adenine- and uridine-based nucleotides seem to be liberated. Attempts to study effects of PNPase on ultraviolet absorption have not yielded any significant observations to date.

Finally, the effects of light (fluorescent and ultraviolet) and temperature upon the absorption spectra of crude extract have been studied (Figure 4). Both light and elevated (room) temperature cause decreases in the absorption spectra at 263 nm. Other changes may be occurring below 240 nm which have not yet been assessed reliably because MOPS buffer absorbs in this region and it can be difficult to duplicate the MOPS concentration (not precisely known) occurring in the sample in the reference cell. Thus, light and temperature conditions appear to be very important factors in this work and may account for the previously unexplained instability of soluble phosphorylation systems. The author has always controlled temperature as is common biochemical practice but until recently had conducted no experiments

to confirm suspicions of photoinactivation.

Outlook

It should be evident from the above discussion that at this stage in the investigation there are many fruitful avenues of research that could be pursued, but one cannot run all directions at once. Since RNase inhibition and light sensitivity of transfer activity are crucial observations, more time must necessarily be spent to assure the reproducibility of this work and to delve into the mechanisms of these effects. Practically everything described in Part II of the results and discussion section requires further work which holds the promise of contributing substantially to comprehension of the RNase and light effects. What has been tried to date should not be viewed as delimiting the techniques which can be brought to bear on this problem, for this would be far from the truth. Nevertheless, each new technique usually requires different equipment and time to become familiar with new procedures.

Further study of the conversion of $^{14}C$Glucose to $^{14}C$G-6-P, DNase versus RNase effects, the relationship of apparent substrate phosphorylations to the complete system, and the $NAD^+$ and CoA effects deserve high priority. The rapid rates of reaction would seem to forecast a need for stopped flow apparatus sometime in the future, but there may be ways to slow the reactions down if necessary. This report has not included all work done since the last report, but that information left out (for example the effect of RNase on an $NAD^+$- or a CoA-stimulated $^{32}Pi$ transfer reaction) may be classed as either still too preliminary to reach any conclusions or else repetitious (may have been done to show reproducibility).

The future at this point appears to be more promising than at any time since Project 2691 was initiated. The X in formulations like A~X and X~P appears from this research to be not only a protein but a ribonucleoprotein. If correct, the involvement of nucleic acids in energy transduction as well as in fields like genetics and protein synthesis should have profound implications for our understanding of life in general. One is tempted to speculate widely on the basis of this discovery, but this is neither the time nor the place.

Table 1

G-6-$^{32}$P ASSAY: N. E. $^{32}$P VERSUS GAS-LIQUID CHROMATOGRAPHY

| Extract | CoA | $^{32}$Pi Incorporation (20 min) | | | |
|---|---|---|---|---|---|
| | | N. E. cpm ± 1% (nanomoles) | GLC cpm ± 3% (nanomoles) | | |
| | | | before | peak | after |
| + | - | 4059 (.080) | 347 | 973 (.144) | 261 |
| - | - | 4470 (.088) | 527 | 267 (.000) | 200 |
| + | + | 8085 (.159) | 691 | 3004 (.540) | 451 |
| - | + | 8536 (.168) | 466 | 327 (.000) | 169 |

Table 2

Comparison of G-6-P Formation from

$^{14}C$Glucose and $^{32}Pi$

| | nanomoles G-6-P formation (20 min.) | |
|---|---|---|
| Stimulator | from $^{14}C$Glucose | from $^{32}Pi$ |
| $NAD^+$ | 41.17 | 0.016 |
| $NAD^+$ + CoA | 47.84 | 0.060 |

Table 3

The Effect of G-3-P on the $^{32}Pi$ Transfer Reaction in the Presence and Absence of $NAD^+$

| Variable* | Incubation Time (min) | cpm/cm peak height in aliquot |
|---|---|---|
| control | "0" | 27 |
| | 20 | 84 |
| $+NAD^+$ | "0" | 391 |
| | 20 | 446 |
| +G-3-P | "0" | 19 |
| | 20 | 143 |
| $+NAD^+$ + G-3-P | "0" | 5334 |
| | 20 | 7187 |
| $+NAD^+$ + G-3-P w/o extract | "0" | 16 |
| | 20 | 4 |

* Incubation system essentially as in Table 5. G-3-P is 3.68 mM; $NAD^+$ is 300 μM.

Table 4

Effects of Washing Mitochondria upon the Unstimulated and Stimulated $^{32}Pi$ Transfer Reation

| Experiment | Variable* | Protein (mg/ml) | Washings | cpm/cm peak height in aliquot Δ≠ | |
|---|---|---|---|---|---|
| 21 | control | 14.6 | 1 | 483 | ( 33) |
| | + $NAD^+$ | 14.6 | 1 | 1786 | ( 122) |
| | + G-3-P | 14.6 | 1 | 1231 | ( 84) |
| | + $NAD^+$ + G-3-P | 14.6 | 1 | 9753 | ( 668) |
| | control | 9.5 | 4 | 598 | ( 63) |
| | + $NAD^+$ | 9.5 | 4 | 4662 | ( 491) |
| | + G-3-P | 9.5 | 4 | 780 | ( 82) |
| | + $NAD^+$ + G-3-P | 9.5 | 4 | 14499 | (1526) |
| 25 | control | 13.5 | 0 | 703 | ( 52) |
| | + $NAD^+$ | 13.5 | 0 | missing | |
| | + G-3-P | 13.5 | 0 | 1133 | ( 84) |
| | + $NAD^+$ + G-3-P | 13.5 | 0 | 38659 | (2864) |
| | control | 15.2 | 1 | 861 | ( 57) |
| | + $NAD^+$ | 15.2 | 1 | 3297 | ( 217) |
| | + G-3-P | 15.2 | 1 | 1673 | ( 110) |
| | + $NAD^+$ + G-3-P | 15.2 | 1 | 68826 | (4528) |
| | control | 9.6 | 2 | 951 | ( 99) |
| | + $NAD^+$ | 9.6 | 2 | 4532 | ( 472) |
| | + G-3-P | 9.6 | 2 | 1208 | ( 126) |
| | + $NAD^+$ + G-3-P | 9.6 | 2 | 87047 | (9067) |
| | control | 6.6 | 4 | 650 | ( 98) |
| | + $NAD^+$ | 6.6 | 4 | 2975 | ( 451) |
| | + G-3-P | 6.6 | 4 | 545 | ( 83) |
| | + $NAD^+$ + G-3-P | 6.6 | 4 | 27842 | (4218) |
| | control | 7.3 | 8 | 550 | ( 75) |
| | + $NAD^+$ | 7.3 | 8 | 1984 | ( 272) |
| | + G-3-P | 7.3 | 8 | 567 | ( 78) |
| | + $NAD^+$ + G-3-P | 7.3 | 8 | 35697 | (4890) |

* Incubation system essentially as in Table 5. G-3-P is 3.68 mM; $NAD^+$ is 300 μM.

Δ ( ) = on per mg protein basis

≠ all 20 min. incubations

Table 5

Control of G-6-P Formation from Pi by RNase, Light, Aging and Exogenous ADP

| Experiment | [a]Variable | [b]cpm/cm peak height/sample | picomoles/cm peak height/sample |
|---|---|---|---|
| A | control | 136,260 | 8.78 |
| | [c]RNase | 120,760 | 7.78 |
| | [d]RNase | 97,500 | 6.28 |
| B | control | 1,478 | 0.33 |
| | [d]RNase | 1,262 | 0.28 |
| | no ADP | 0 | 0.00 |
| | Aged in light | | |
| | 30 min. at room temp. | 312 | 0.07 |
| | Aged in dark | | |
| | 30 min. at room temp. | 786 | 0.18 |
| C | control | 708 | 1.00 |
| | [e]RNase | 94 | 0.13 |
| | no ADP | 142 | 0.20 |
| D | control | 30,240 | 6.80 |
| | [e]RNase | 20,900 | 4.70 |
| | no ADP | 21,960 | 4.94 |
| | Aged in light | | |
| | 30 min. at 4° | 9,020 | 2.03 |
| | Aged in dark | | |
| | 30 min. at 4° | 16,440 | 3.70 |

[a] The components of the 4 ml incubation system were: MOPS, pH 7.2, 50 mM; $MgSO_4$, 1.5 mM; Pi, 10 μM; ADP (where added), pH 6.8, 100 μM; glucose, 2.5 mM; hexokinase (P-L grade 300), 30 units; $^{32}Pi$ specific activities of 7,764 (A), 4,484 (B), 710 (C), and 4,444 (D) cpm/picomole. Reactions were initiated by addition of 1 ml of mitochondrial extract and incubated for 4 min. at room temp. G-6-P (1 mg/ml) carrier present during incubations of experiments B and C.

[b] cpm + 3% or better

[c] Worthington crystalline, 25 μg/ml

[d] Worthington crystalline, 250 μg/ml

[e] Nutritional Biochemicals crystalline, 250 μg/ml

Figure 1

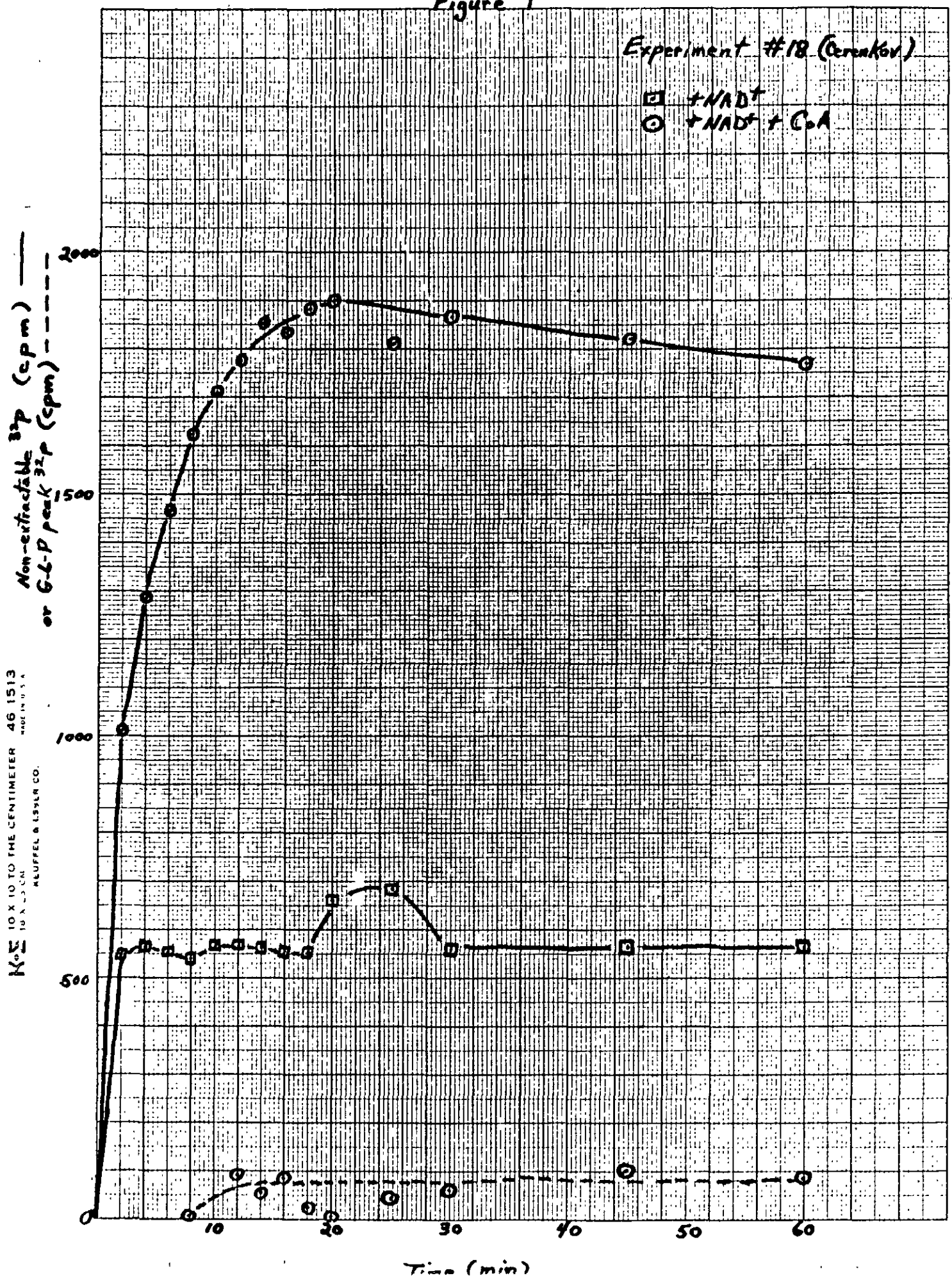

Experiment #18 (Cerenkov)
+NAD+
+NAD+ + CoA
Non-extractable 32P (cpm) ——
or 6-L-P peak 32P (cpm) - - - - -
2000
1500
1000
500
0
10
20
30
40
50
60
Time (min)
K&E 10 X 10 TO THE CENTIMETER 46 1513
KEUFFEL & ESSER CO.

Figure 2

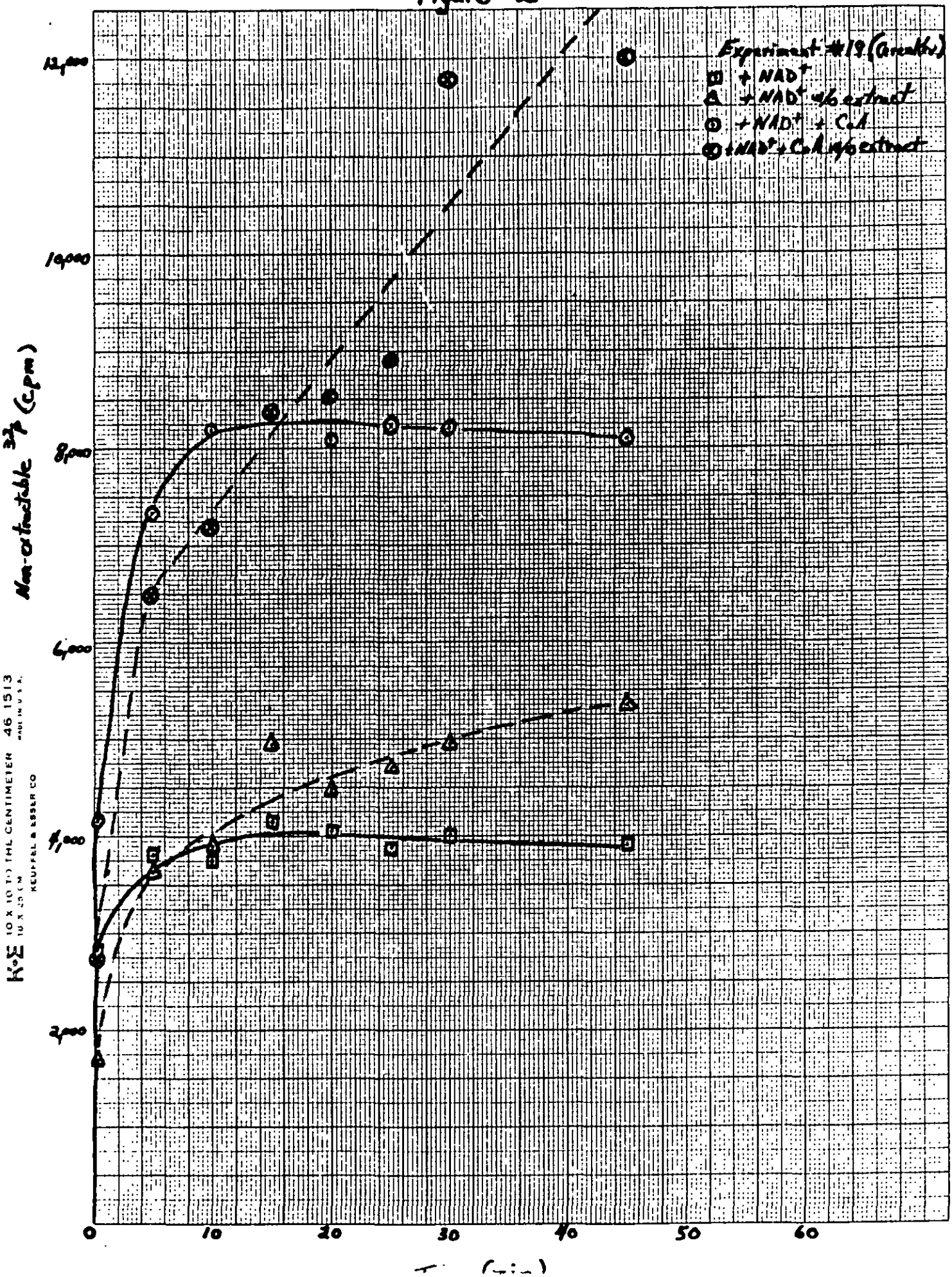

Experiment #19 (Annette)
□ + NAD+
△ + NAD+ +/6 extract
○ + NAD+ + CoA
⊗ + NAD+ + CoA +/6 extract
Non-extractable 32P (cpm)
12,000
10,000
8,000
6,000
4,000
2,000
0
10
20
30
40
50
60
t (min)

Figure 3

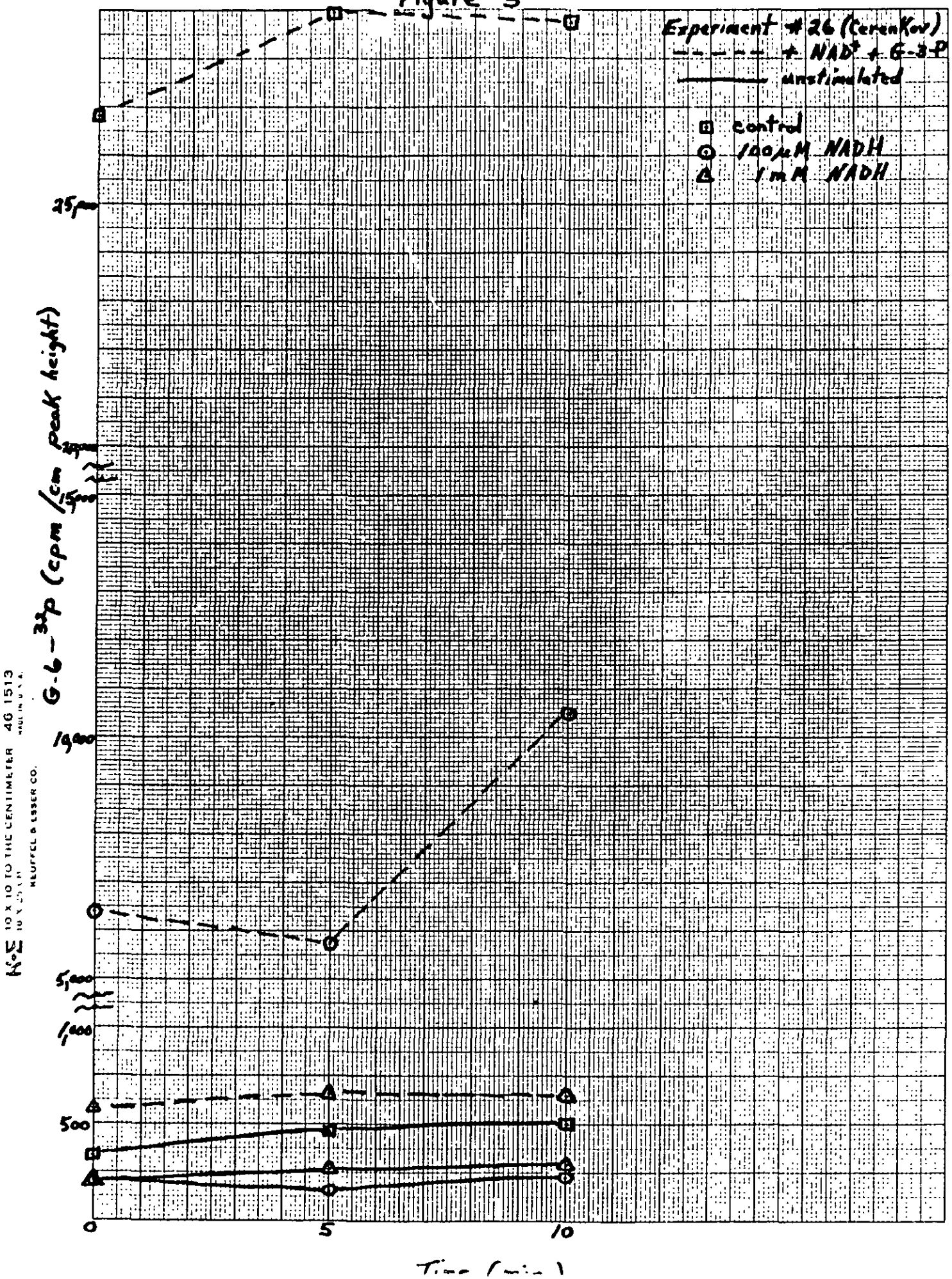

Experiment #26 (CerenKov)
+ NAD+ + G-3-P
unstimulated
control
100 μM NADH
1 mM NADH
G-6-32P (cpm / cm peak height)
25,000
15,000
10,000
5,000
1,000
500
0
5
10
Time (min)

Figure 4

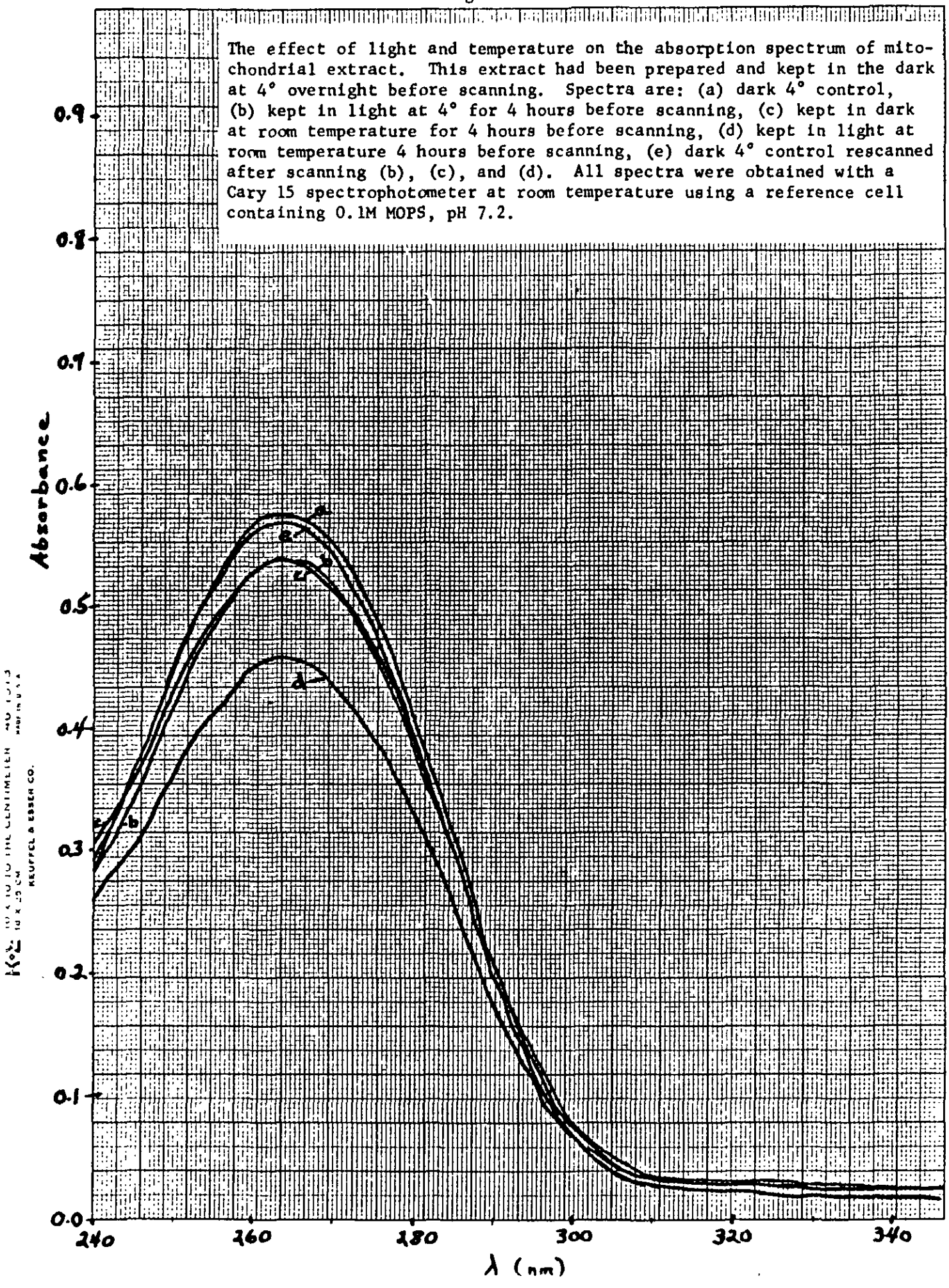

The effect of light and temperature on the absorption spectrum of mitochondrial extract. This extract had been prepared and kept in the dark at 4° overnight before scanning. Spectra are: (a) dark 4° control, (b) kept in light at 4° for 4 hours before scanning, (c) kept in dark at room temperature for 4 hours before scanning, (d) kept in light at room temperature 4 hours before scanning, (e) dark 4° control rescanned after scanning (b), (c), and (d). All spectra were obtained with a Cary 15 spectrophotometer at room temperature using a reference cell containing 0.1M MOPS, pH 7.2.
Absorbance
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
240
260
280
300
320
340
λ (nm)
a
b
c
d
e

Figure 5

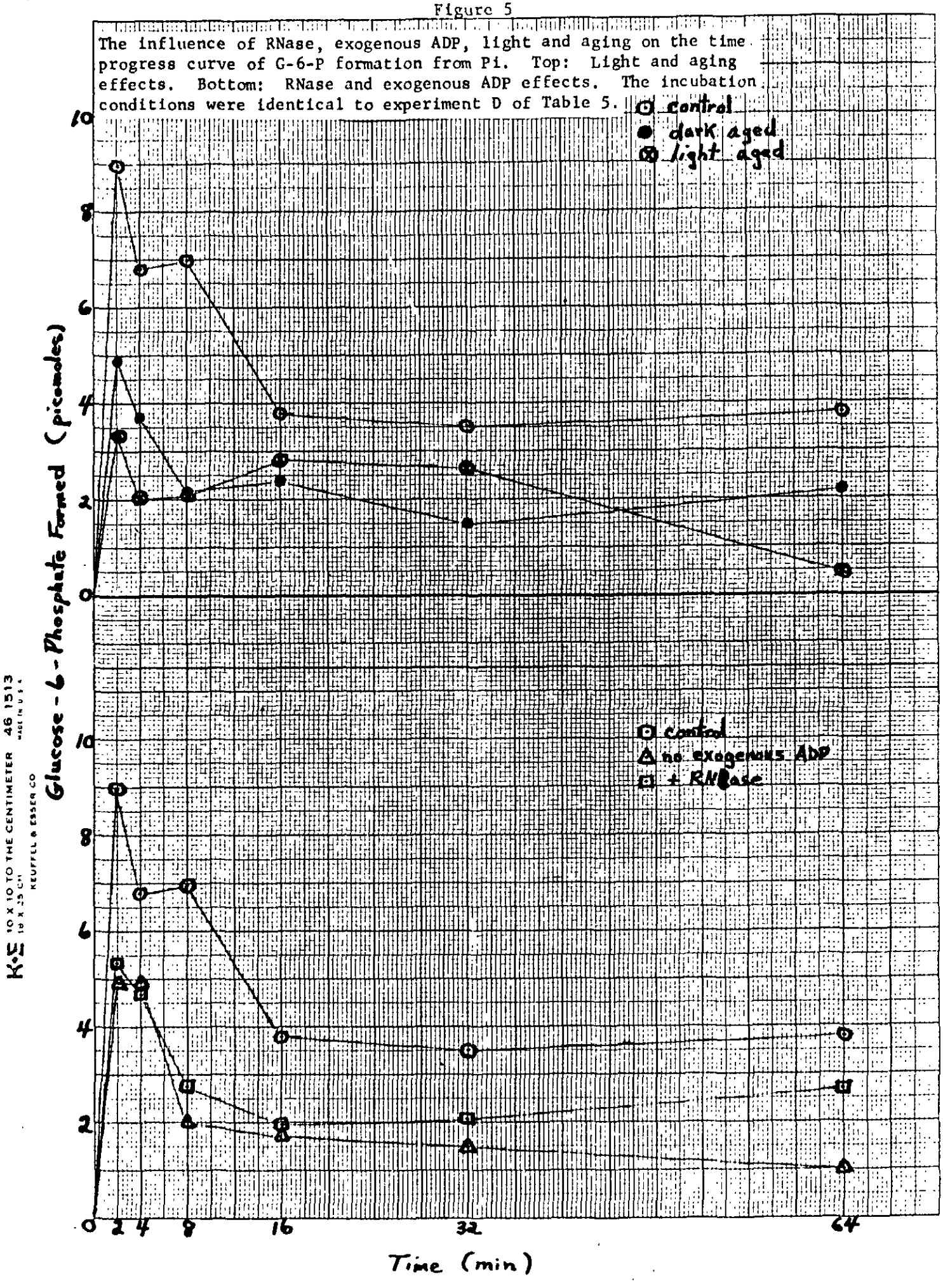

The influence of RNase, exogenous ADP, light and aging on the time progress curve of G-6-P formation from Pi. Top: Light and aging effects. Bottom: RNase and exogenous ADP effects. The incubation conditions were identical to experiment D of Table 5.
control
dark aged
light aged
Glucose-6-Phosphate Formed (picomoles)
control
no exogenous ADP
+ RNase
0
2
4
8
16
32
64
Time (min)
K&E 10 X 10 TO THE CENTIMETER 46 1513
KEUFFEL & ESSER CO

Figure 6

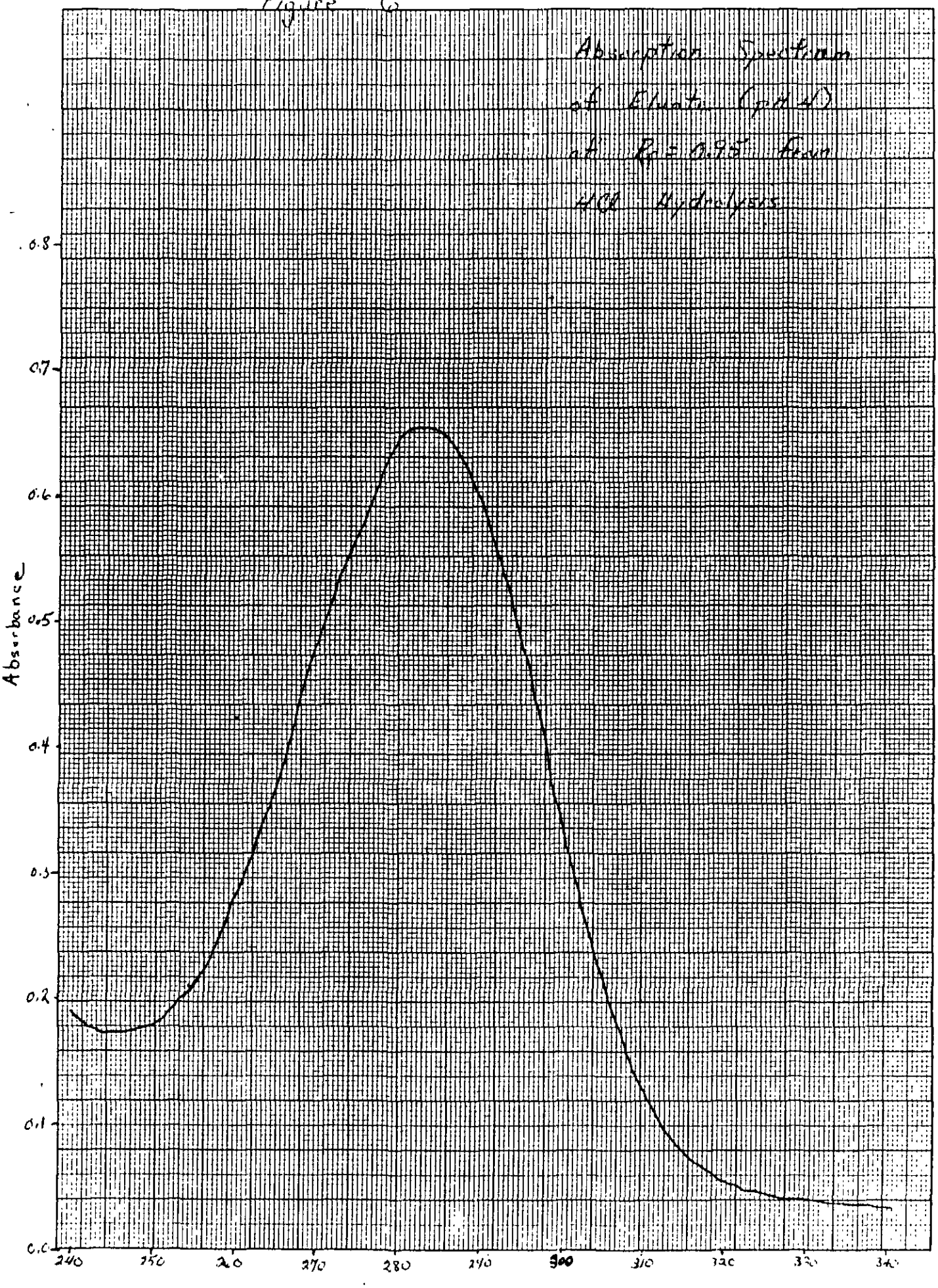
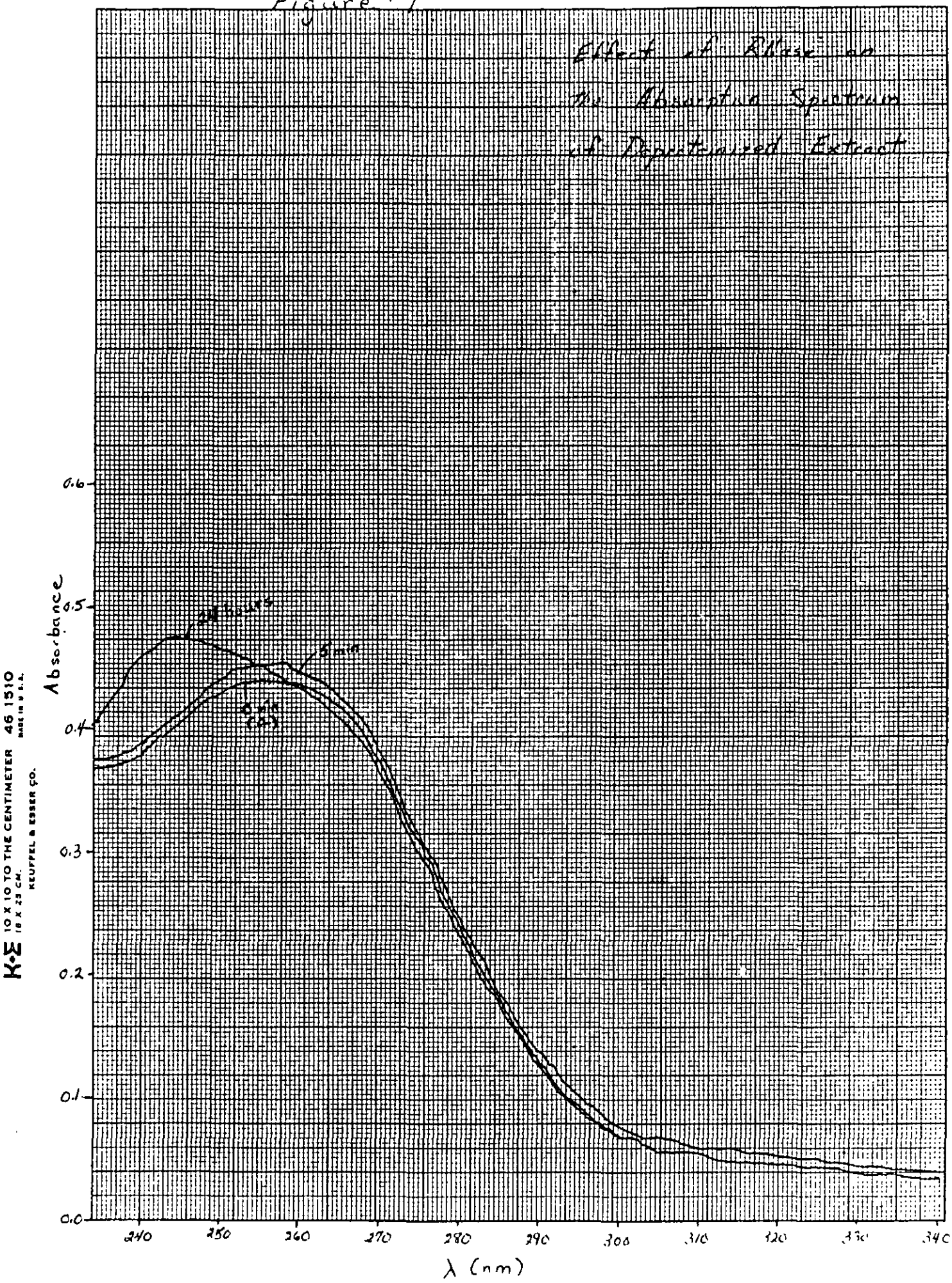

Absorption Spectrum
of Eluate (pH 4.0)
at $R_f$ = 0.95 from
40% Hydrolysis
Absorbance
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
240
250
260
270
280
290
300
310
320
330
340
KEUFFEL & ESSER CO.
MADE IN U.S.A.

Figure 7

Effect of Filtration on the Absorption Spectrum of Deproteinized Extract



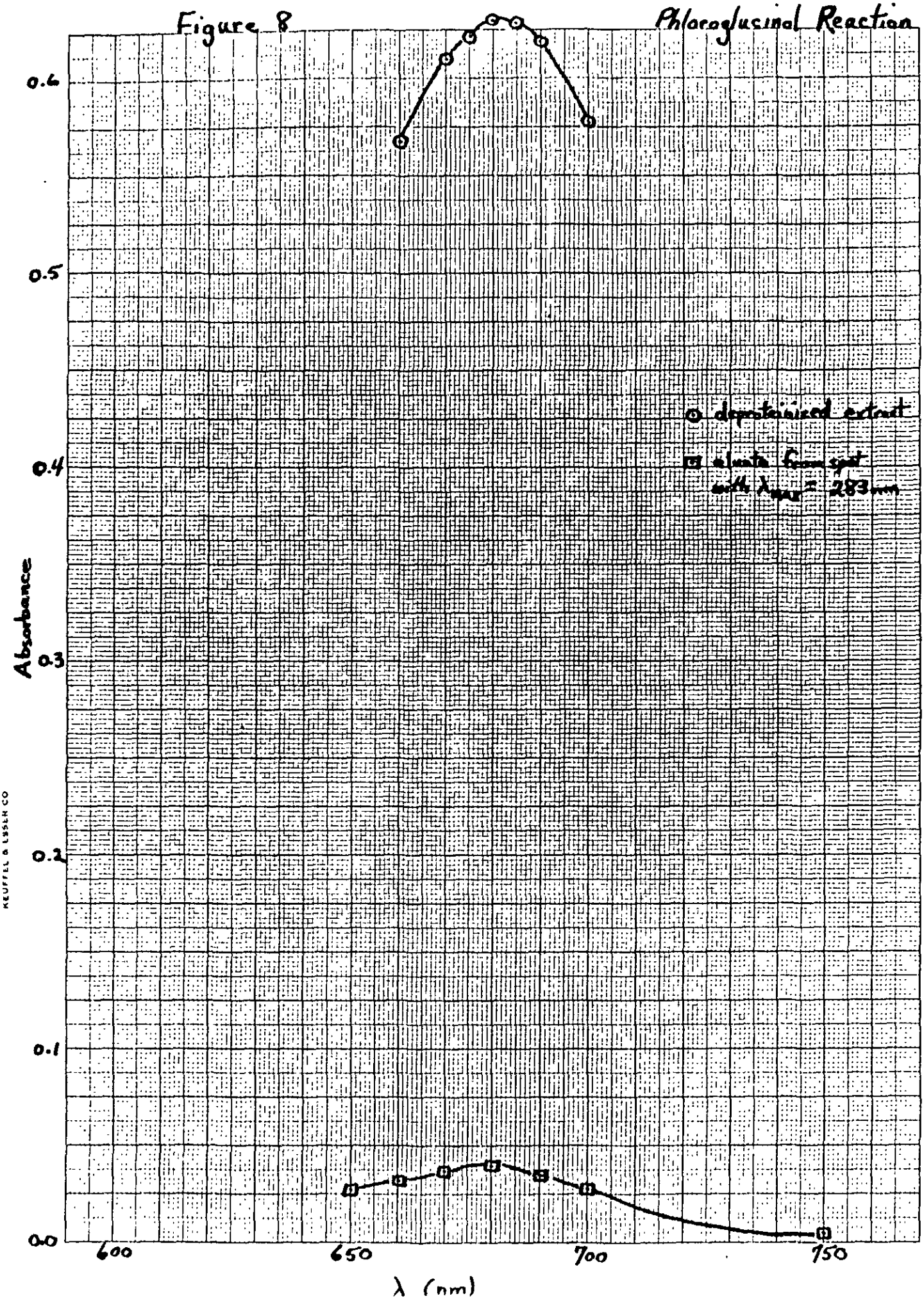
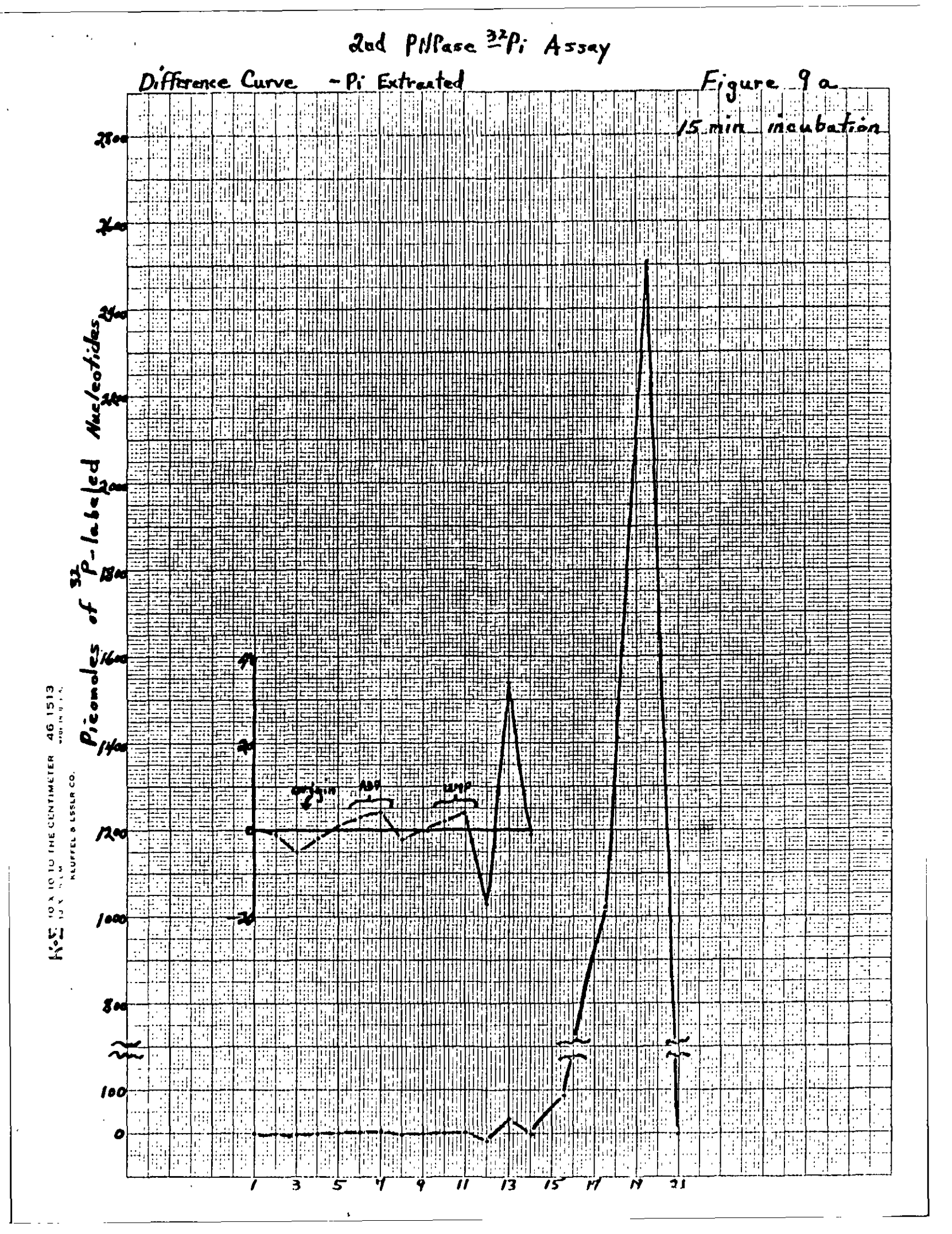


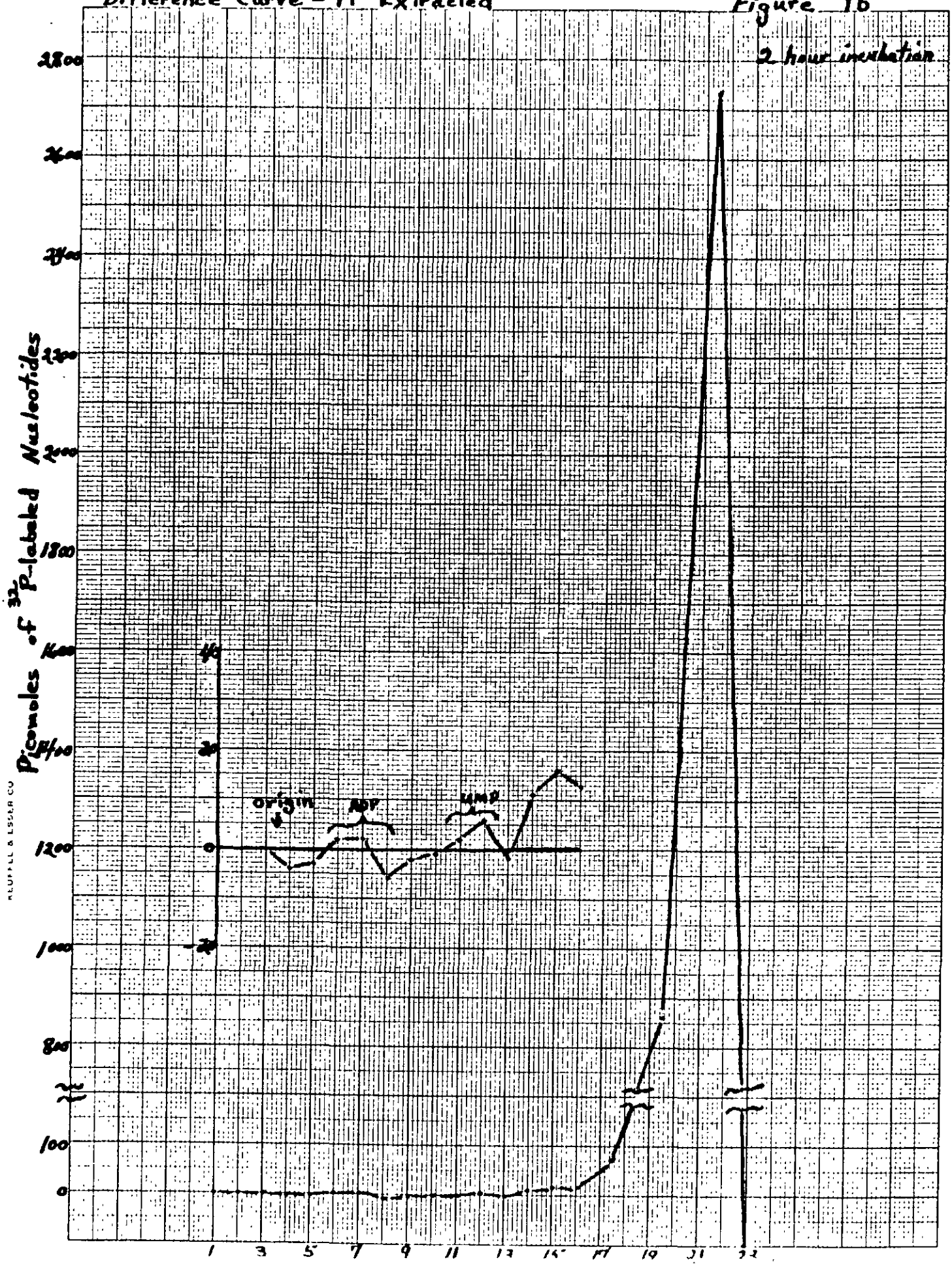
Figure 8
Phloroglucinol Reaction
0.4
0.5
0.4
0.3
0.2
0.1
0.0
Absorbance
600
650
700
750
λ (nm)
⊙ deproteinized extract
⊡ eluate from spot
with λmax = 283 nm
KEUFFEL & ESSER CO


2nd PNPase 32Pi Assay
Difference Curve -Pi Extracted
Figure 9a
15 min incubation
Picomoles of 32P-labeled Nucleotides
2800
2400
2000
1800
1600
1400
1200
1000
800
100
0
-20
20
40
origin
ADP
ATP
1
3
5
7
9
11
13
15
17
19
21

2nd PNPase – $^{32}P_i$ Assay

Difference Curve – $P_i$ Extracted

Figure 9b



## References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(Plant Bioenergetics)
Project Report (1)

# PROJECT REPORT FORM

PROJECT NO. 2691
COOPERATOR Institute of Paper Chemistr
REPORT NO. 4
DATE August 25, 1971
NOTE BOOK 2743 pp. 138-160 & 2871
PAGE 1 TO 71
SIGNED Morris A. Johnson

Copies to: Files
Swanson
Einspahr
M. A. Johnson (3)
Carlson
Weiner

## MECHANISMS IN BIOENERGETICS OF PLANTS


### SUMMARY

Time progress curves for G-6-P formation as G-6-$^{32}$P and $^{14}$C G-6-P have similar shapes characterized by sharp rise to a maximum followed by subsequent apparent disappearance of G-6-P. Contrary to previous interpretations, it is now virtually certain that the destruction of G-6-P is real and probable that the responsible enzyme(s) are contaminants of the commercial hexokinase employed. RNase inhibition curves follow the same pattern, but RNase appears to be less effective if added beyond time zero, suggesting that its effect is not on G-6-P disappearance. Recent characterization studies of the ATP-Pi exchange reaction indicate that it may not be succinic thiokinase. Attempts to separate cleanly transfer and exchange activities have not been successful to date.


### INTRODUCTION

Experiments conducted during this report period* have been of several different types. The use of both $^{14}$C-Glucose and $^{32}$Pi consistently revealed the presence of a soluble system capable of G-6-P formation via ATP generation. Inhibition of G-6-P formation by ribonuclease (RNase) has been observed regardless of whether the radioactive label is initially attached to glucose as $^{14}$C or to Pi as $^{32}$P; however, there is some evidence that only Pi-stimulated $^{14}$C G-6-P formation is susceptible to RNase, suggesting a possible differentiation between phosphorylated (X~P) and nonphosphorylated (A~X) intermediates. The

*Essentially since November, 1970; shortly after report No. 3 there was a serious equipment breakdown leading to inactivity.

classical uncoupler of oxidative phosphorylation, 2,4-dinitrophenol (DNP) has also been found to be effective against the formation of $^{14}C$G-6-P as well as G-6-$^{32}P$.

Analytical procedures continue to confirm the presence of nucleic acid in the mitochondrial extracts. Nevertheless, it still remains unknown whether in fact the RNA present is essential for the ATP generation, i.e., that RNase is an inhibitor because it attacks RNA.

Attempts to publish what is known have met with several split decisions by reviewers. In the most recent attempt the Canadian Journal of Biochemistry left the door open to resubmit this work as a full length paper. Since this is the second journal to suggest that this research should not be published as a preliminary communication, there is considerable impetus for submission of a major paper. However, to do this, it would be desirable to have the RNA involvement much more detailed than it is since it seems to be too radical an idea for many reviewers to accept without extensive documentation. It was contemplated that a major paper entitled "Partial Purification and Properties..." could be submitted if some purification of the crude extract could be realized. Efforts to this end in recent months have resulted in limited progress and, in fact, renewed the question whether transfer and exchange activities are properties of separate entities. The methodology involved in this research and new developments with the exchange reaction may provide alternative publication routes.

There has been no response to the publication of the RNase inhibition observations in Bioenergetics Bulletin. In some heartening related developments, there have been several publications indicating that the existence of classical chemical intermediates is still a very viable hypothesis and that

the alternative chemiosmotic and conformational hypotheses are losing experimental support in the sense that they are conceived by Mitchell and Green respectively [e.g. see Biochemistry 10 2897 (1971)]. Also, correspondence has been established with some French workers who have been investigating the role of thiols in oxidative phosphorylation. The properties of the cabbage intermediates seem to be consistent with their whole mitochondria work (1) with respect to thiol involvement. The coenzyme A (CoA)-dependent succinate-stimulated ATP-$^{32}$Pi exchange reaction which accompanies the ATP-generating system in the cabbage extract may not be succinic thiokinase activity after all. Combining the observations and hypotheses of the French investigators with two publications (2, 3) by Roberts A. Smith in California in 1965, one can visualize an alternate explanation for this ATP-$^{32}$Pi exchange.

RESULTS AND DISCUSSION

Acetone in crude extract - In January of this year an investigation was conducted into the nature of the UV absorbant material responsible for a large included peak when crude extract was passed through Biogel P-6. This material had been examined previously (see project report No. 2 pp. 8-9) but remained a puzzle since the analytical report on a freeze-dried fraction suggested that it must be inorganic. It was known that this material was not essential for transfer or exchange activity, but it had to be investigated because of its influence on any UV work. The failure of the included peak material to react with orcinol turned attention back to acetone despite the fact that the UV peak was at 263 nm. Acetone was a known contaminant of the crude extract but, if one checks its UV spectrum in the literature $\lambda$max close to 274 nm. are always listed; furthermore, the absorption near 260 nm. always made nucleotides suspect. However, nucleotides should react with orcinol. Summarily, it was finally realized that acetone in aqueous solutions has a lower $\lambda$max

and known 0.5% acetone in double-distilled water was found to have an identical $\lambda$max with the P-6 included peak samples and approximately the same extinction. Since samples previously examined by the analytical department had been lyophilized, it is easily seen why they weren't able to locate any organic material.

This discovery fortunately did not have serious repercussions since material excluded from P-6 is still active, is orcinol positive and has a relatively low 280/260 absorbance ratio. Nevertheless, it has focused attention on the acetone to the extent that some work conducted on crude extract should be repeated. For example, acetone appears to play a role in the stability of the system. P-6 excluded material does not show visible denaturation upon aging as quickly as crude extract, especially at room temperature where the organic solvent is apt to be more damaging. By and large, the presence of 0.5% acetone would have little or no significance in most work, but results based upon UV examination, in particular, need confirmation on P-6 excluded material.

RNA involvement - Crude extract has been found to give the same $\lambda$max as known yeast RNA with both the orcinol and phloroglucinol reagents. With the exception of one special sample, the phloroglucinol absorption maxima of knowns and samples have consistently been at 630 nm. rather than at 680 nm. (literature value). A slight reaction with diphenylamine (DNA reagent) has also been noted. The reagents of the Sevag procedure used to separate protein from nucleic acid have been found to not interfere with the orcinol test, thereby validating data which shows that extract deproteinized via the Sevag procedure is orcinol positive. Acetone does not interfere either, and P-6 excluded extract is orcinol positive.

The Sevag procedure has been used to prepare a protein-free extract from which RNA could be precipitated by cetyltrimethylammonium bromide (CTAB), a very efficient RNA precipitant (4). Material was precipitated by CTAB but the process needs further investigation since it appeared to be less efficient than it was hoped. According to the author of the method, the nucleic acid is not denatured and can be recovered after precipitation. It is anticipated that this procedure may facilitate future work on the RNA component. More chemical hydrolyses and enzymatic phosphorolysis followed by paper chromatography of resultant products have been carried out, but, since acetone may be interfering in this work, interpretation will be delayed pending work on P-6 excluded extracts. Incidentally, the inclusion of P-6 beads in the extraction medium, which has been a batch process for acetone removal during extraction, has been discontinued since it is not very efficient and can occasionally lead to difficulties in centrifugation.

Kinetic experiments were conducted with $^{32}Pi$ labeling in an attempt to fathom the reason for the apparent disappearance of G-6-$^{32}P$ product (i.e., as in figure 5 of report No. 3). It was reasoned in the last report that the disappearance of G-6-P in these figures may be more apparent than real, i.e., one may be simply observing a drop in the specific activity of G-6-$^{32}P$ due to dilution from a source of unlabeled phosphorus. However, as one reviewer pointed out, it is conceivable that G-6-P is really disappearing and, therefore, the possibility exists that the RNase effect may be exerted at that level rather than upon ATP formation. In the two experiments shown in figures 1 and 2 the effect of RNase addition at zero time versus RNase addition at 60 seconds was investigated. If the RNase effect were on ATP formation as proposed, one might expect it to be effective only when added at $t = 0$ and not at $t = 60$ seconds when ATP formation should be nearly complete.

Vice-versa, if RNase accelerates G-6-P breakdown, one might expect RNase to be more effective at later stages of the reaction and its time of addition to be noncrucial. In the experiment of figure 1 the RNase addition at t = 0 led to results consistent with effects on ATP formation but the behavior of control and RNase at t = 60 seconds did not permit definite conclusions since the data appeared somewhat erratic and curves crisscrossed. An attempt to expand and repeat this experiment is shown in figure 2. To confuse the issue, in this experiment a lag period was encountered which was slightly longer in the control. Note that there is an overall similarity in the results of the two experiments including curve shapes and an apparent "stimulation" of G-6-$^{32}$P yield when RNase is added late. The trend in the data would seem to favor the limited conclusion that RNase added late is a relatively ineffective inhibitor. It is very difficult to run these experiments because there is no way to predict the kinetic behavior of the control in a very exact way; therefore, one can only approximate when the late RNase addition should be made. At this stage such experiments have been inconclusive and have yielded a new puzzle piece requiring a fit (RNase "stimulation" when added late).

After confirming previous observations on the capacity of the extract to produce relatively large amounts of $^{14}$C G-6-P when U-$^{14}$C-Glucose was used instead of $^{32}$Pi for labeling (Table 1, cf. Table 2 of report No. 3), a time progress curve was also obtained for this system (Figure 3). Some quenching problems were encountered with $^{14}$C but, when these were overcome, the control curve was very similar to the $^{32}$P results. Some variables including inhibitors gave very interesting results as shown but complete time progress curves for each of these conditions should be obtained before serious interpretation can be made. The results indicate complete enzyme and ADP dependence and stimulation by unlabeled Pi in confirmation of earlier results. The Pi-stimulated

$^{14}C$G-6-P yield is reduced about 20% by the presence of RNase and somewhat more by DNP. Tentatively, it may be only the Pi-dependent portion of the $^{14}C$G-6-P yield which is RNase-sensitive, suggesting that A~X (measured by $^{32}Pi$ and also by the difference between Pi-stimulated and unstimulated $^{14}C$Glucose incorporations) is susceptible but not X~P (measured by $^{14}C$Glucose incorporation in the absence of added Pi). Perhaps the most revealing aspect of figure 3 is the shape of the curve which shows $^{14}C$G-6-P disappearing just as does G-6-$^{32}P$. While the previous mechanism advanced for this disappearance of G-6-$^{32}P$ (report No. 3 p. 13) is not necessarily inoperative, it can't be used for the $^{14}C$G-6-P case. Therefore, it seems inescapable that $^{14}C$G-6-P really is destroyed in the system (if so, the same conclusion must be extended and hold for most if not all G-6-$^{32}P$ disappearance also) and, considering the stability of G-6-P, there is probable enzyme catalysis of the destruction process. An inquiry is being sent to P-L Laboratories that supply the hexokinase to confirm that G-6-P Dehydrogenase along with a few other enzymes is present in their hexokinase in very small amounts even though this is probably one of the best sources of purified hexokinase available. Although it may be a formidable task to rigorously purify hexokinase, it has potential to solve not only this problem but simultaneously to eliminate previous frustrations in efforts to examine contaminating enzymes in the mitochondrial extract. It may be possible to purchase ultrapure hexokinase on special order but the costs are apt to be prohibitive.

Fractionation attempts - The initial experiment to further explore salt fractionation as conducted several years ago found transfer and exchange activities distributed as expected, but the separation was not clean (Table 2). With renewed awareness of the acetone content of extracts, some variations of salt fractionation methodology were then attempted such as complete precipitation

with salt first (leaving acetone behind) followed by reconstitution and subsequent fractionation. These efforts were not very encouraging but not all combinations of light and temperature have been examined.

Since several pieces of evidence* indicated that highly charged molecules were under investigation, the possibilities of fractionation by ion exchange were probed. Preliminary to use of any ion exchangers the pH stability of the extract and its affinity for Sephadex ion exchangers was examined. Commencing at neutral pH, titration with base produced little information while titration with acid led to slight turbidity below pH 6.5 which slowly changed to white precipitate as the pH was lowered further. This white floc was most conspicuous at about pH 5.3 at which point this material must be isoelectric. As the pH was lowered further this floc went back into solution, leaving a few floating threadlike particles in its wake. The threads, once-formed, seemed to be permanently insoluble while the white floc could be regenerated on raising the pH again. Extract was also batch-treated with DEAE Sephadex (weak anion exchanger ), CM Sephadex (weak cation exchanger) and SE Sephadex (strong cation exchanger). Binding to exchangers was determined by UV absorption measurements on and acid titration of unadsorbed extract (supernatants after treatment and centrifugation). Results are summarized in Tables 3 and 4. Although DEAE Sephadex appears to be the best choice, it formed a white flocculent precipitate with MOPS buffer in the range of pH 7-9.5 which made its use hazardous. In these trial runs it was possible to re-elute material bound to CM Sephadex by raising the pH. Thus some attempts were made to use CM Sephadex in both batch and column forms. It was not thought likely that it would be effective in adsorbing the active material but that it

*Especially a special studies investigation which attempted fractionation on two types of gel filtration supports.

might remove contaminating material. CM Sephadex has been tried in fractionation experiments but, as can be seen in Tables 5 and 6, it has not resulted in good separation of transfer and exchange activities. Transfer activities in CM eluates were too weak to generate much hope that they were real, especially since there was a rarely encountered high blank for transfer activity in the batch experiment.

Exchange characteristics - The most recent experiment was an investigation of what has heretofore been referred to as succinic thiokinase (STK) exchange activity. The very good data obtained (Table 7) cast doubt whether this really was STK exchange and, if not, whether it was within the realm of possibility to separate it from transfer activity of which it might be part and parcel. In at least some respects the exchange reacts to perturbations like transfer does and, it seems to have none of the properties of STK from other sources as reported in the literature. Despite the fact that stimulation by DNP is seen here, it is likely from older experiments that inhibition would have appeared with longer incubation times. DNP interference with ATP formation is characteristic of oxidative phosphorylation but not substrate phosphorylation (STK). Exchange inhibition may require higher concentrations of DNP and longer incubations, but these exchange assays measure only turnover not amounts of material reacting as in the transfer assay. More convincing, neither transfer nor exchange are sensitive to atractyloside which should have wiped out STK exchange at the concentrations that I have used (5). Most intriguing is the fact that so far (higher concentrations must be tried) pantetheine has not been able to substitute for CoA in the exchange at concentrations equal to or 10x the CoA concentration used. If it were really STK pantetheine should work (2). Smith's CoA - dependent ATP-Pi exchange enzyme shares this characteristic

with the cabbage extract exchange, and it is also DNP sensitive. However, Smith found that with his enzyme the terminal bridge oxygen in ATP originated from Pi contrary to Boyer's evidence that ADP supplies this oxygen atom in oxidative phosphorylation (3,6). It must be pointed out that Smith used a soluble enzyme from a microorganism while Boyer used intact rat liver mitochondria, leaving room for possible species differences. Also, Boyer's data may not be ironclad even though it has been widely accepted for several years; certainly it was obtained from a more complex system.

Stimulation rather than inhibition of exchange by RNase is seen in Table 7 also. This may be a case like that of DNP. If one compares this result with the behavior of RNase in figure 2, it is sensed that there is a possibility of stimulation prior to inhibition and that the true picture at any given concentration may require several time points. One must proceed very cautiously in the interpretation of this data, however, for past experience has shown that increases in nonextractable $^{32}P$ are not necessarily increases in $AT^{32}P$ nor enzymatic in origin. Thus, in the future nonenzymatic controls must be run not only for the complete system but also with each specific complement of inhibitors and other added reagents. Ultimately, one must proceed to specific ATP analysis for those changes which are enzymatic. This has been done for the complete system through termination of exchange by freezing followed by addition of unlabeled ATP and lyophilization. The lyophilized sample was then reconstituted and treated with the hexokinase trap to form G-6-$^{32}P$ and refrozen (no unlabeled G-6-P was then added as would be customary in transfer assays). After lyophilization again the sample was derivatized and gas chromatographed with collection and counting of the G-6-$^{32}P$.

MISCELLANEOUS

There have been a few research efforts which do not fit neatly into the above headings but are worth mentioning. Attempts to cut down the amount of $^{32}Pi$ trailing on the gas chromatography of G-6-$^{32}P$ were not successful. The use of molybdate to complex excess $^{32}Pi$ prevents the subsequent formation of the Tri Sil derivative of G-6-P. Precipitation of excess Pi as $MgNH_4PO_4$ may have some promise but would be unwieldy for routine work.

The dichromate assay for total organic matter (7), briefly used previously in Project 2396, was adapted for use on cabbage extract. It has given substantially higher values than are obtained as protein concentrations using the UV absorption measurements, even with the acetone contribution eliminated. This observation is in agreement with other evidence that nonprotein macromolecules are in the extract (e.g. RNA).

OUTLOOK

At this juncture in the research it seems possible to focus future work quite clearly. However, the execution of experiments which should be done is expected to encounter a good many problems in the acquisition of good data.

The following areas should be attacked:

1. The purity of commercial hexokinase. It is hoped that it may be possible to solve this problem by purchase of hexokinase completely free from G-6-P degrading enzymes. If not, the attempt will have to be made to purify commercial hexokinase in very small quantities here at the Institute. If the purification can be accomplished, it should allow publication of previous data in which G-6-P ($^{14}C$ or $^{32}P$ labeled) disappearance was manifest. In my opinion it should not be necessary to use ultrapure hexokinase for all of this research

but it should be sufficient to demonstrate that hexokinase is the source of the contaminating enzymes.

2. Further study of the characteristics of the ATP-Pi exchange reaction. Data such as shown in Table 7 need to be confirmed and investigated in more detail in line with the discussion which accompanied that table . In the case of pantetheine substitution for CoA, some experiments with whole cabbage mitochondria would seem to be necessary to establish an internal point of reference rather than relying solely on the literature for succinic thiokinase characteristics. Firm evidence that this exchange is not succinic thioknase would lessen and possibly eliminate the pressure to separate it from transfer activity.

3. Further work on fractionation with ammonium sulfate. The question to be answered is whether or not a clean separation of transfer and exchange is possible. It is a complex issue because possibilities exist that lack of activity in a given fraction may represent inactivation rather than fractionation and that exchange may be detectable at levels where transfer is not, even though it is still present. Nevertheless, it is far too soon to concede a standoff in this area.

4. Analysis of RNA in the ribonucleoprotein. Of particular interest in this area are:

(a) CTAB precipitation of RNA from deproteinized extract, followed by recovery, hydrolysis, and chromatographic examination of products.

(b) Further chromatographic examination of products from RNase attack upon crude and/or deproteinized extract.

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TABLE 1

G-6-P Formation from $^{14}C$Glucose

| System | cpm/cm. peak height (4 min.) |
|---|---|
| complete acceptor system | 42 |
| complete acceptor system + Pi | 51 |
| acceptor system - ADP | 0 |
| (complete acceptor system + Pi w/o enzyme (control)) | 0 |

TABLE 2

$(NH_4)_2SO_4$ Fractionation

| Fraction | Protein mg. (280/260) | Activities cpm/cm. peak height Transfer | Transfer + $NAD^+$ | Transfer + C.A | cpm Exchange |
|---|---|---|---|---|---|
| original extract | 11.01* | 646 | 982 | 1045 | 391,640 |
| 0% ppt. | 1.65 | 166 | 123 | 184 | 26,370 |
| 0-75% ppt. | 2.10 | 155 | 161 | 332 | 106,630 |

*acetone contributes to this figure

TABLE 3

Ultraviolet Absorbance by Extract Unbound to Ion Exchangers

| Samples | *Absorbance (uniform dilution basis) 280 nm. | 260 nm. | 280/260 |
|---|---|---|---|
| original extract | 0.560 | 0.746 | 0.75 |
| DEAE supernatant | 0.360 | 0.490 | 0.73 |
| CM supernatant | 0.408 | 0.548 | 0.74 |
| SE supernatant | 0.428 | 0.578 | 0.74 |
| 0.1M MOPS, pH 7 | 0.050 | 0.070 | 0.71 |

*small constant acetone contribution to all but buffer data

TABLE 4

Acidification Behavior of Ion Exchange Supernatants

| Ion Exchanger | Description of Supernatant Behavior |
|---|---|
| SE Sephadex | threads by pH 6.0 with some cloudiness; both threads and white floc at pH 5.0; white floc redissolves by pH 2.6 |
| CM Sephadex | similar to SE Sephadex |
| DEAE Sephadex | no threads by pH 6.15; nothing by pH 5.5 except a few threads; little change by pH 5.15 and pH 2.5 |
| 0.1M MOPS | no change |

TABLE 5

Batch Fractionation with CM Sephadex

| Fraction | Protein mg. (280/260) | transfer activity cpm*/cm. peak height | exchange activity cpm* |
|---|---|---|---|
| P-6 eluate | 0.516 | 63 | 483,762 |
| Unbound to CM | 0.395 | 77 | 169,463 |
| CM eluate | 0.102 | 31 | 0 |

*above no enzyme blank

TABLE 6

Column Fractionation with CM Sephadex

| Fraction | Protein mg. (280/260) | transfer activity cpm*/cm. peak height | exchange activity cpm* |
|---|---|---|---|
| P-6 eluate | 0.586 | 155 | 133,363 |
| Unbound to CM | 0.576 | 111 | 166,232 |
| CM eluate | 0.163 | 13 | 0 |

*above no enzyme blank

TABLE 7

Some Characteristics of the ATP-$^{32}$Pi Exchange

| Assay System (Exchange) | Counting Data** external standard | cpm | ±% counting error |
|---|---|---|---|
| Complete, no enzyme control | 5.20 | 200 | 3 |
| Complete | 5.21, 5.27 | 5567, 5836 | 3, 3 |
| Complete - CoA | 5.25, 5.29 | 200, 161 | 3, 5 |
| Complete - succinate | 5.30, 5.26 | 375, 317 | 3, 3 |
| Complete + DNP | 5.20, 5.22 | 6846, 6489 | 3, 3 |
| Complete + Atractyloside | 5.15, 5.24 | 6452, 6550 | 3, 3 |
| Complete + RNase | 5.16, 5.23 | 7072, 7173 | 3, 3 |
| Complete - CoA + lo Pantethine + DTT* | 5.19, 5.22 | 209, 180 | 3, 5 |
| Complete - CoA + hi Pantethine + DTT* | 5.20, 5.25 | 162, 174 | 5, 5 |
| Complete + DTT* | 5.33, 5.17 | 7642, 6814 | 3, 3 |

*Dithiothreitol (DTT) is used to reduce pantethine to pantetheine *in situ*

**Duplicate except control

Figure 1

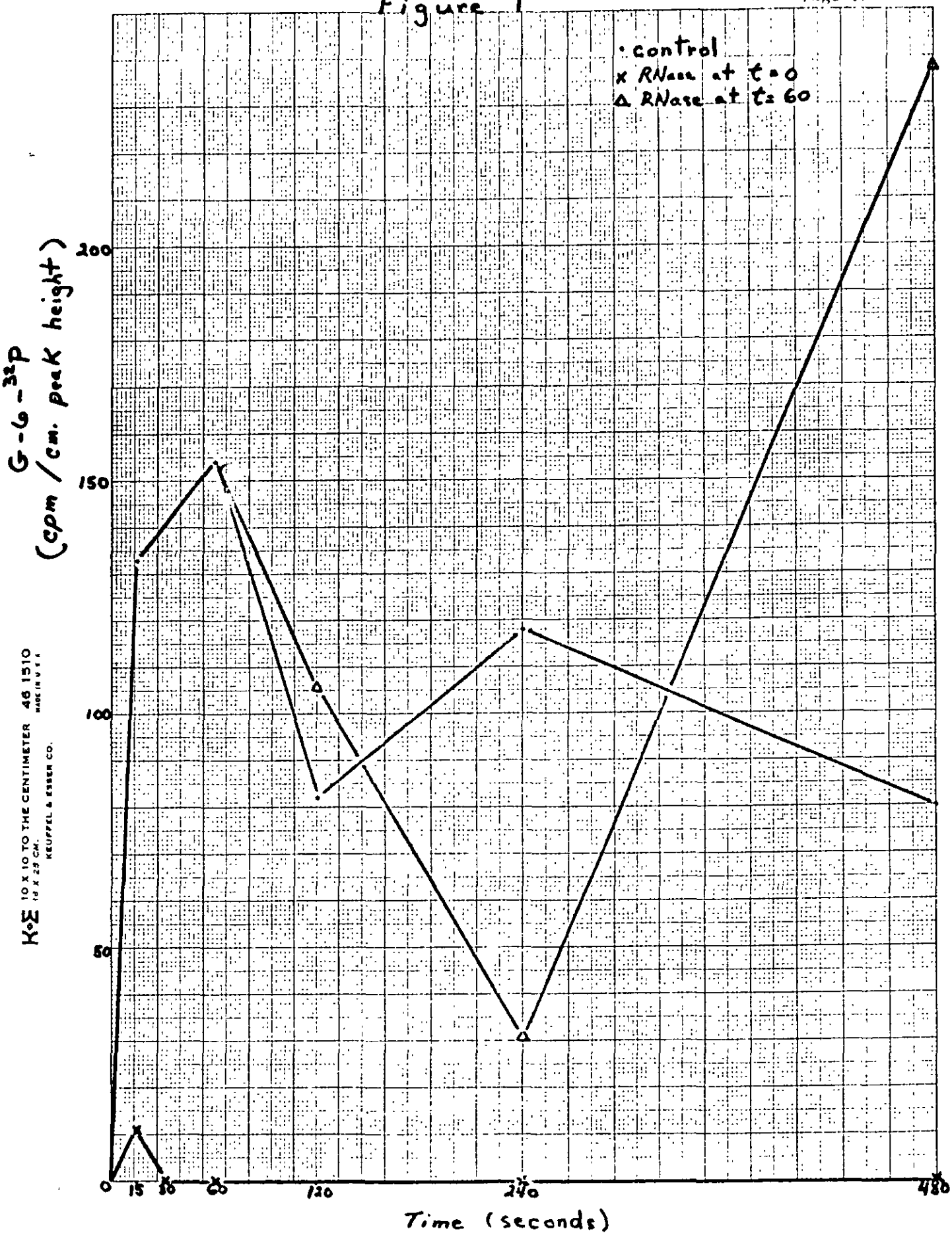

· control
x RNase at t = 0
Δ RNase at t = 60
G-6-32P (cpm / cm. peak height)
200
150
100
50
0
15
30
60
120
240
480
Time (seconds)
K&E 10 X 10 TO THE CENTIMETER 46 1510
KEUFFEL & ESSER CO.

Figure 2

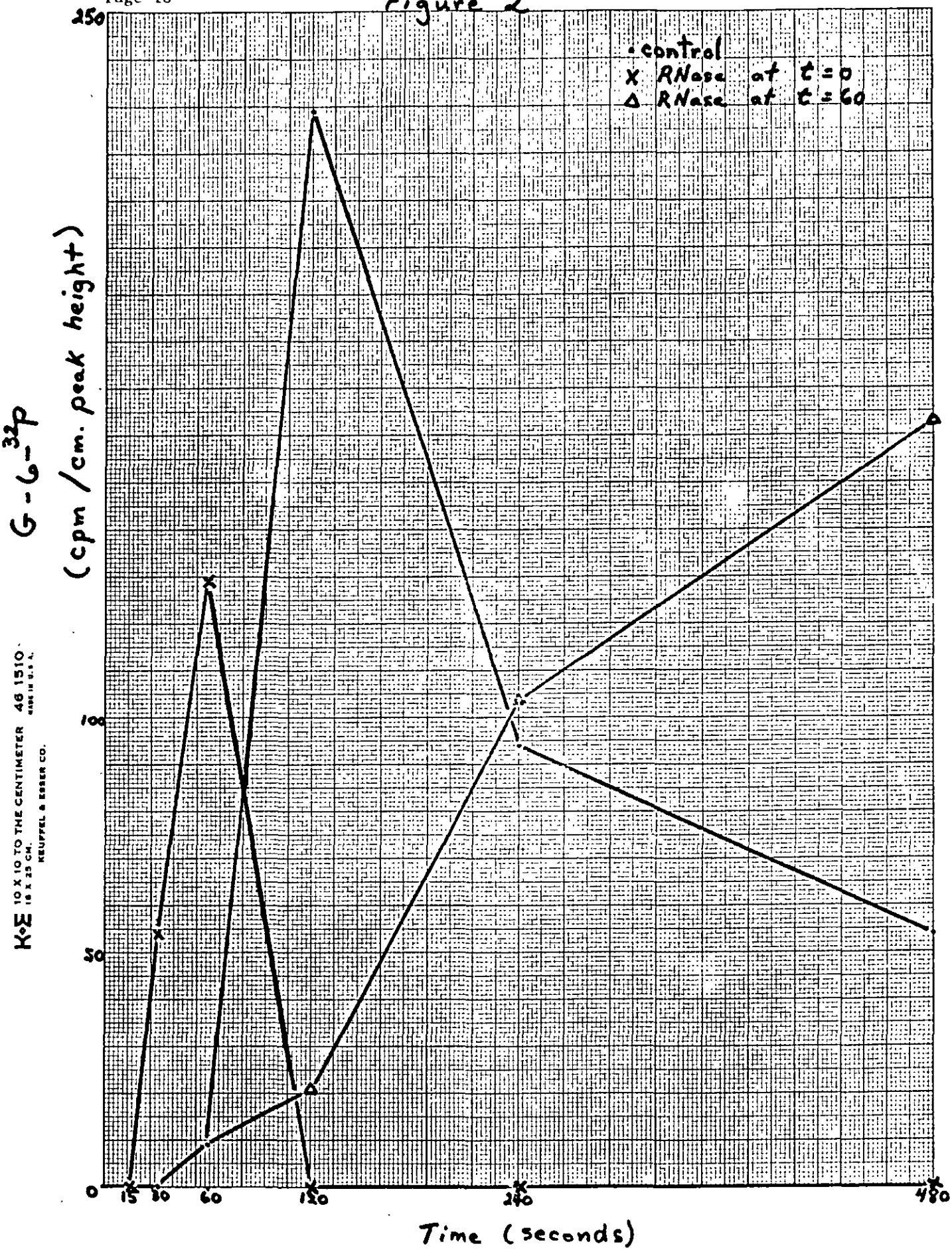
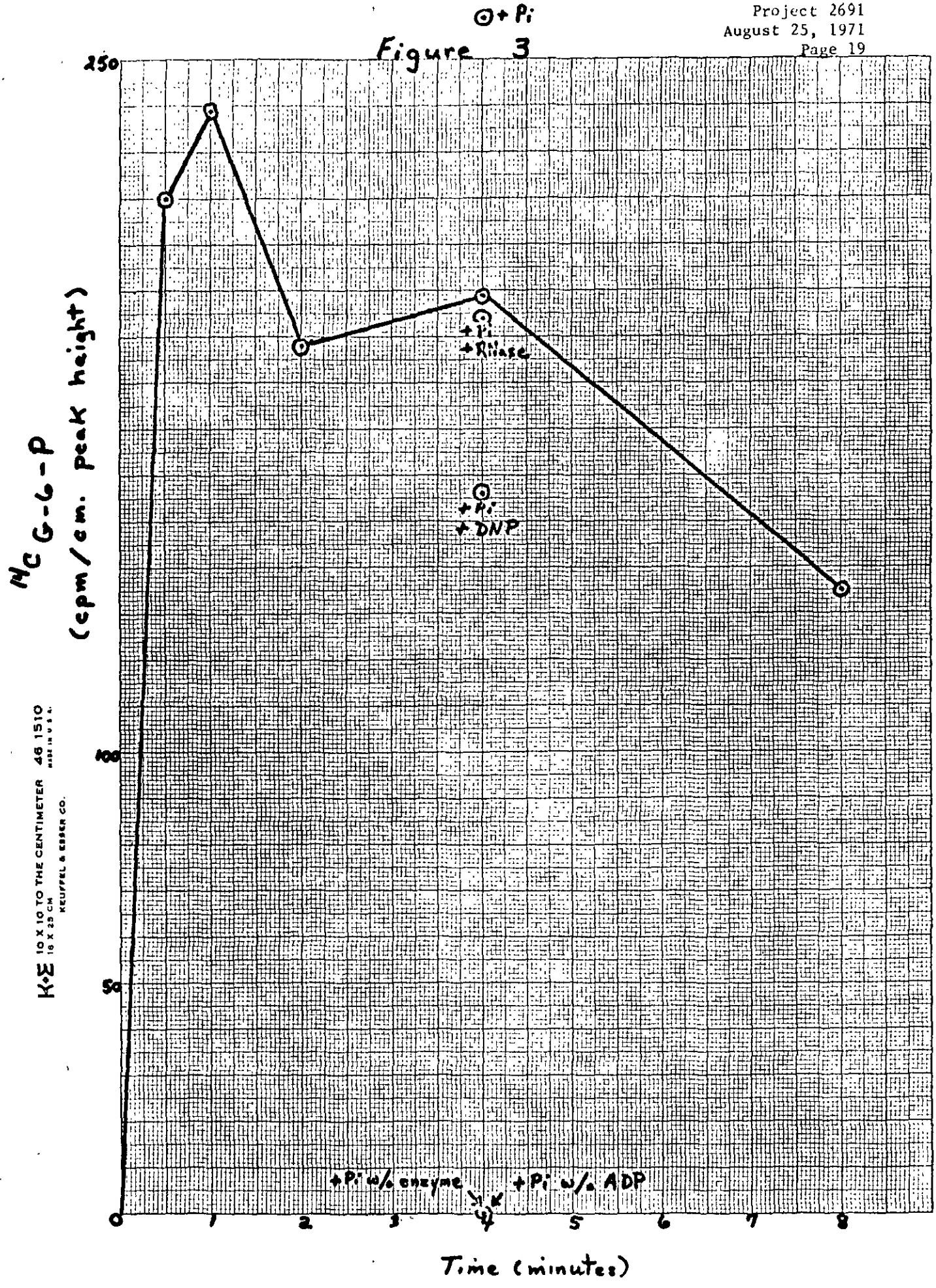

250
100
50
0
15
30
60
120
240
480
• control
X RNase at t = 0
Δ RNase at t = 60
G-6-32P (cpm/cm. peak height)
Time (seconds)
K&E 10 X 10 TO THE CENTIMETER 46 1510
18 X 25 CM.
KEUFFEL & ESSER CO.

Figure 3


⊙+ Pi
250
200
50
0
1
2
3
4
5
6
7
8
+ Pi
+ Riase
+ Pi
+ DNP
+Pi w/o enzyme
+ Pi w/. ADP
Time (minutes)
$N_{C_{G-6-P}}$ (cpm/cm. peak height)
K·E 10 X 10 TO THE CENTIMETER 46 1510
16 X 25 CM
KEUFFEL & ESSER CO.